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## PREFACE

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## CONTENTS

	Page
1. INTRODUCTION .....	9
2. MATERIALS .....	10
2.1 Patch Microprobe Instrumentation .....	10
2.2 Recording Chambers .....	11
2.3 Computer Analysis .....	12
2.4 Channel-Forming Test Substances .....	15
3. METHODS .....	15
3.1 Planar Phospholipid Bilayers .....	15
3.2 Manufacture of Patch Micropipettes .....	15
3.3 Methods of Forming an Artificial Biomembrane Patch .....	19
3.4 Reconstitution Trials with Muscle Homogenates and Sonicates .....	20
3.5 Channel-Forming Test Substances .....	20
3.6 Cholinergic Drug Trials .....	21
3.7 Sources and Study of Anion Channels .....	22
3.8 Activity of Acetylcholinesterase on Silicon Monoxide Substrates .....	24
4. RESULTS .....	25
4.1 Lipid Compositions .....	25
4.2 Channel-Forming Test Substances .....	28
4.3 Cholinergic Drug Trials .....	35
4.4 Sources and Study of Anion Channels .....	36
4.5 Activity of Acetylcholinesterase on Silicon Monoxide Substrates .....	39
4.6 Muscarinic Receptors on Cardiac Myocytes .....	39
5. CONCLUSIONS .....	40
LITERATURE CITED .....	43

## LIST OF FIGURES

1. Schematic of the Instrumentation for the Patch Microprobe System .....	10
2. Recording Chamber Designs .....	12
3. Histogram of Single-Channel Durations from a Gramacidin-Containing Patch Phospholipid Bilayer .....	14

4.	Typical Single-Channel Amplitude Histogram for Gramacidin Channels in a Patch Phospholipid Bilayer .....	14
5.	Current-Voltage Curve for Gramacidin in a Lecithin-Sphingomyelin Patch Bilayer .....	18
6.	Current-Voltage Curve for Nonactin in a Sphingomyelin Patch Membrane .....	21
7.	Current-Voltage (I-V) Curve for Alamethacin in an Artificial Patch Biomembrane .....	22
8.	Microforged Micropipette .....	23
9.	Diagrammatic View of How Antibiotic Molecules Form Channels in Planar Phospholipid Bilayers .....	30
10.	Discrete Channel-Like Events in a Phospholipid Bilayer Induced by Gramacidin .....	31
11.	Single-Channel Event in a Phospholipid Bilayer Induced by Gramacidin .....	32
12.	Transmembrane Conductance Changes in the Presence of d-tubocurarine and Gramacidin .....	33
13.	Curare-Induced Conductance Alterations Across a Patch Phospholipid Bilayer in the Presence of Gramacidin .....	34
14.	Curare-Induced Discrete Fluctuations in Membrane Conductance .....	35
15.	Conductance Shifts Induced by Nonactin Across a Patch Phospholipid Bilayer .....	36
16.	Nonactin-Induced Conductance Changes in Artificial Patch Membranes .....	37
17.	Voltage-Dependent Conductance Fluctuations Across Patch Phospholipid Membranes in the Presence of Amphotericin B .....	38
18.	Unverified Chloride Channel Activity in Reconstructed Patch Membranes .....	39

## LIST OF TABLES

1.	Comparison of Different Glass Capillary Tubing for the Manufacture of Patch-Type Micropipettes/ Microelectrodes .....	16
2.	Tests of Potential Patch Membrane-Forming Lipids .....	27

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## ELECTROPHYSIOLOGICAL AND ELECTROCHEMICAL METHODS DEVELOPMENT FOR THE DETECTION OF BIOLOGICALLY ACTIVE CHEMICAL AGENTS

### 1. INTRODUCTION

The present investigation at the University of Nevada includes work done in two interwoven projects: the development of electroimmunoassay probes as a self-contained analytical device to monitor electrically the concentration of biochemicals; and the investigation of electrophysiological detection characteristics of chemical agents in reconstituted and artificial excitable membrane preparations. The present contract focused primarily on the development of a microprobe system using patch-clamp technology with artificial lipid bilayer membranes, and also on the capacitance film (silicon monoxide) technology under development at the Johns Hopkins University Applied Physics Laboratory (JHU/APL). The muscarinic receptor was studied more than the chloride channel because of the importance of this receptor in a biosensor scheme for known threat agents.

The goals of this 2-year investigation are to:

- a. Develop an artificial membrane model system using lipid planar bilayers in which protein receptors can be inserted.
- b. Develop a computerized microprobe system using patch-clamp technology to study the electrophysiological and pharmacological characteristics of receptors inserted into the artificial biomembranes.
- c. Examine the feasibility of applying the microprobe system to a biosensor detector system for reliable and routine detection of cholinergic drugs and potential threat agents.
- d. Explore the feasibility of using muscarinic receptors of mammalian origin in the microprobe biosensor system.
- e. Study the radioenzymatic activity and pharmacological characteristics of acetylcholinesterase bonded to the surface of silicon monoxide substrates provided by JHU/APL.

Some of the work highlights of the past year include the formation of a bilayer detected by the channel-like conductance fluctuations induced by gramicidin, ionic conductances due to cytotoxic T lymphocyte target cell [P815(H-2d mastocytome of DBA/2)] interactions, improvements made in the pulling of patch micropipettes, and research on an ideal new synthetic lipid compound, L-alpha-phytanoyl lecithin (Avanti).

## 2. MATERIALS

### 2.1 Patch Microprobe Instrumentation.

The basis of the microprobe system is an AxoPatch Patch-Clamping Amplifier System (Axon Instruments, Inc.). This is a specialized electronic voltage-clamping system for tight-seal patch clamping for single-channel and whole-cell recording. It is an advanced instrument with many convenient features built in. Figure 1 represents a schematic of the apparatus required for the microprobe system as implemented in this laboratory. Current and voltage outputs from the AxoPatch are fed to an oscilloscope; the Tecmar Labmaster interface board associated with the Compaq (MS-DOS) computer system; and, if required for backup, an X-Y electronic recorder or an optional frequency-modulated tape recorder or auxiliary Smartscope Transient recorder.

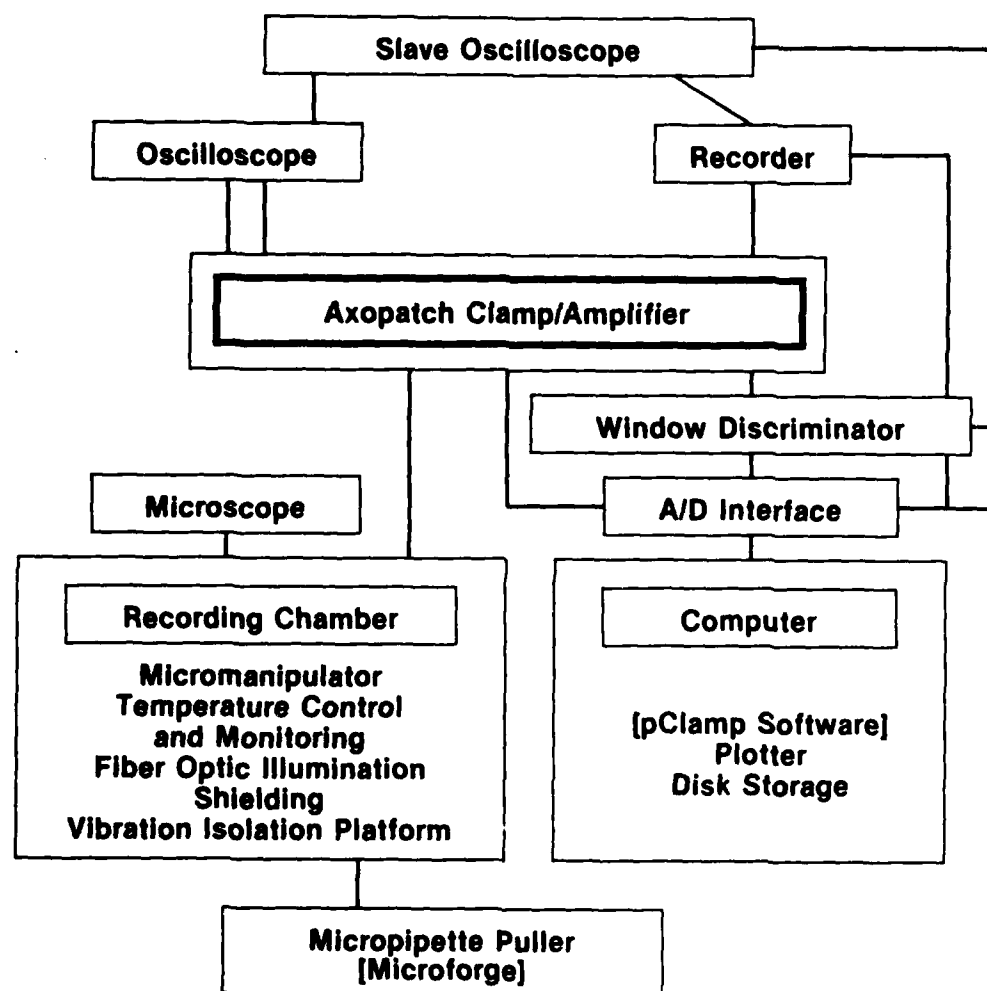


Figure 1. Schematic of the Instrumentation for the Patch Microprobe System

Severe low-pass filtering of the outputs is conventional for patch-clamping experiments; a 2-kHz cutoff or, in extremely noisy conditions, a 0.5- to 1-kHz cutoff was routinely used. The recording platform consists of an active-air vibration isolation unit surrounded on three sides and above with a "Ferro cage" shielding system. The micromanipulators are magnetically mounted on the steel surface of the floating tabletop. The area is illuminated with fiber optic light tubes to prevent introduction of 60-cycle interference. A Peltier thermoelectric heat exchanger (Cambion) is magnetically mounted to the tabletop to allow precise control of temperatures in the recording chamber; its direct-current power supply sits close to the floor to minimize interference. Careful shielding, filtering of the mains, and grounding considerations have virtually eliminated electromagnetic and radio-frequency interference from the apparatus. Nonetheless, a perforated aluminum screen on magnet mounts shields the face of the recording area during experiments.

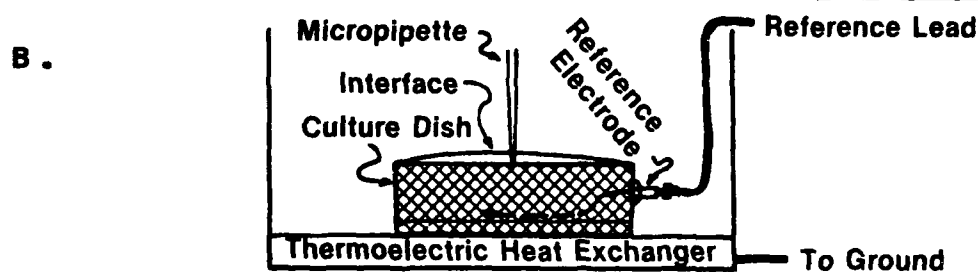
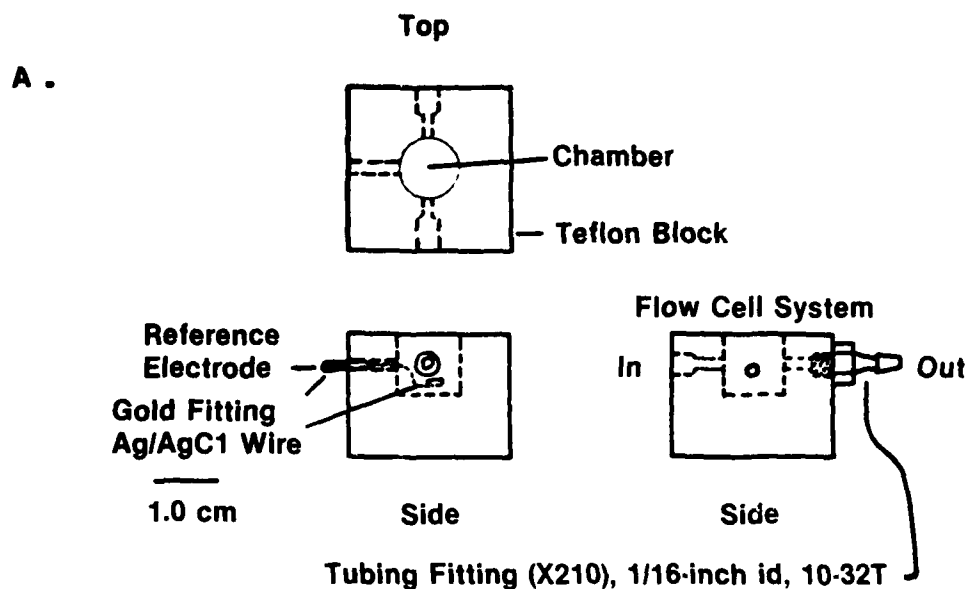
## 2.2 Recording Chambers.

Several Teflon block chambers (Figure 2) were designed and manufactured. Falcon (1008) plastic disposable culture (Petri) dishes (35 x 10 mm) are also favored because no cleaning procedures are required, and they have intrinsically low noise potential. Multiwell plastic Elisa trays also work well but require repeated micromanipulation of the reference electrode as the recording cells are advanced. Essentially, any small volume vessel (not subject to electrical noise, perhaps of a capacitance nature) is acceptable.

Space is only needed to insert the AgCl-Ag wire reference electrode into the medium to complete the recording-clamping circuit. The Teflon block recording chambers are fitted with an inlet and an outlet connection so that a flow-cell system for changing media is created. The plastic culture dishes do not work as well for exchanging media due to their larger volume. Screening experimentation may be best accomplished in a modified multiwell tray assembly, although we have not attempted this.

The only guiding principle for chamber design regards contamination and subsequent cleaning. Any contamination, especially oils, fats, or dust, will compromise the formation of a pure monolayer and may contaminate the surface of the reference electrode. Thus, the Teflon block chambers must be thoroughly cleaned after each use. The chambers and attached AgCl reference electrode are cleaned with organic solvents such as chloroform, methanol (2:1), acetone, hexane, or ethanol.

Unfortunately, most plastic chambers will deteriorate or melt when cleaned with such organic solvents. Therefore, disposable plastic cups appear to be the most practical solution. Fisherbrand Autoanalyzer sample cups (Stock No. 02-554-65) with a volume of 0.25 mL are particularly adaptable.



Panel A represents a chamber designed as a flow cell and constructed from Teflon block.

Panel B illustrates a simple recording chamber utilizing a plastic disposable culture dish (Falcon 1008; 35 × 10 mm). The dish is overfilled so that the surface of the medium where the monolayer is formed becomes convex (exaggerated in this illustration). The reference electrode access port is sealed with silicon rubber sealant. The dish is placed in a metal specimen can connected to ground to form an electrical interference shield around the recording area.

Figure 2. Recording Chamber Designs

Chambers were electrically shielded for recording by placing them in the bottom of a shallow metal can connected to a ground; or they were mounted on the thermoelectric heater unit and shielded with heavy aluminum foil connected to a ground.

### 2.3 Computer Analysis.

Computer analysis of transmembrane conductance fluctuations has been unnecessary for much of the preliminary work implementing the microprobe system and testing various lipid bilayer compositions, membrane stability, temperature dependence, and channel-forming activity. However, in preparation for the testing of receptors coupled to ionic conductance modules within the membrane (i.e., receptor-regulated, channel-like structures), a software package called pCLAMP (Axon Instrumentation, Inc.) has

been incorporated into the system. This system is designed as a generic patch-clamping electrophysiological data acquisition and analysis package and is compatible with the AxoPatch System. The pCLAMP software package resides in a Compaq DeskPro Model IV MS-DOS computer with a 20-Mb hard disk and communicates with the other experimental apparatus via a Tecmar Labmaster interface card and rack-mounted daughterboard/BMC connectors panel.

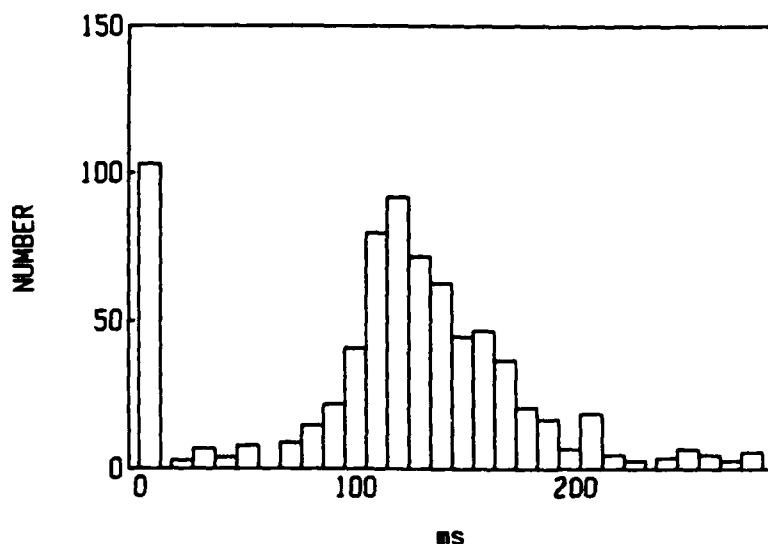
In brief, pCLAMP supports two types of experiments--those that are interval-driven under command of the computer and those that are event-driven by events in the biological preparation. For interval-driven experiments, the computer may be programmed to generate a sequence of command voltages while it simultaneously records the responses evoked by, for instance, ramping through a series of step voltages during a voltage-clamp experiment. The event-driven routines wait for a random event or trigger to occur in the membrane, such as a single-channel opening. The investigator sets an internal or external time and amplitude window discriminator for the event of interest.

The occurrence of an event that satisfies the window parameters triggers the computer to acquire the event and hold it in memory. Numerous analyses can be done during data acquisition if the computer is fast, or the data or critical portions of the data can be stored on a magnetic medium for subsequent analysis at the end of the trial or experiment.

pCLAMP contains four major programs that are of interest to the present project: CLAMPEX for interval-driven voltage-clamp experiments, CLAMPAN for analysis of CLAMPEX data, FETCHEX for event-driven experiments on spontaneous events, and FETCHAN for analysis of FETCHEX data. The use of CLAMPEX and CLAMPAN is anticipated when the patch-clamping parameters are established for routine screening and testing of receptive materials sent by other contractors and in studies of the muscarinic receptor. FETCHEX and FETCHAN will be used in the analysis of single-channel activity derived from receptor-agonist interactions.

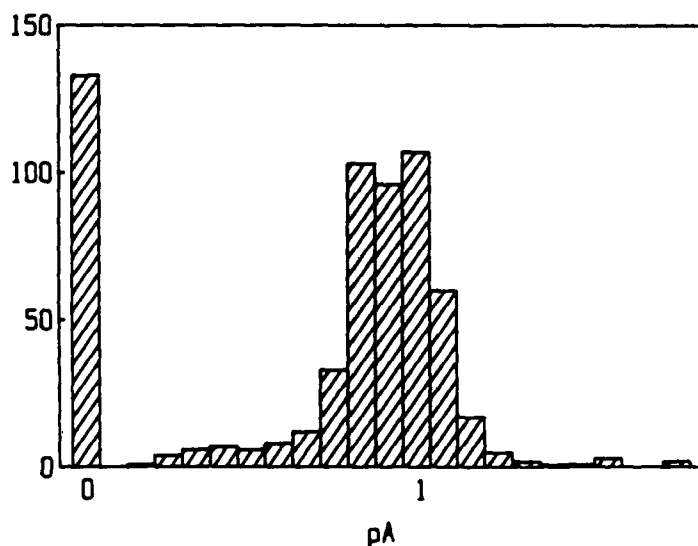
Parameters such as single-channel durations (open state) will be plotted on histograms (Figure 3) as a means of characterizing receptive materials during a pharmacological challenge and under changing intramembrane environments. Histograms of single-channel amplitude distributions (Figure 4) will also be important for comparing receptor-agonist interactions and detecting alterations in the artificial biomembrane.

For much of the preliminary work, example signals were recorded on a Smartscope transient recorder-waveform analyzer system or on a Bascom Turner X-Y electronic recorder. Either of these devices allows a more detailed analysis of waveforms than is possible in comparison with visual monitoring of sweeps on a normal oscilloscope.



This is a typical histogram generated by computer (pCLAMP software) in which 1024 events were sampled and distributed according to temporal duration. A window discriminator detected a channel-like event across a preset threshold triggering the computer to measure the interval of time until the channel-like event turned off (cross the negative slope threshold). Durations of 400 ms or longer were not counted. The bar near zero represents high-frequency electrical noise spikes and can also be eliminated by external or digital filtering if necessary. Histograms of this nature can be used to discriminate the activity of dissimilar receptive moieties in the same membrane, alterations in the activity of receptor-associated ionic channels, and/or can signal modifications in the properties of the membrane due to temperature, solvent, drug, or ionic changes.

Figure 3. Histogram of Single-Channel Durations from a Gramacidin-Containing Patch Phospholipid Bilayer



This is a computer-generated amplitude histogram of 1024 events cut above 2 pA. Events sampled are thought to be from the same single gramacidin channel in a lecithin: sphingomyelin (5:1) patch membrane and is supported by the Poisson distribution about a single amplitude. The bar near zero represents unfiltered noise occasionally crossing the detection threshold. This is difficult to eliminate without missing real-channel events; excess filtering may skew the data. Amplitude histograms are important discriminator tools for studying ionic channel alterations by drugs, environments, or chemical agents. Shifts in amplitude with no change in general membrane resistance (or conductance) signal interactions of an agent with the receptor-associated channel, or separate independent channel when not associated with a receptor complex.

Figure 4. Typical Single-Channel Amplitude Histogram for Gramacidin Channels in a Patch Phospholipid Bilayer

## 2.4 Channel-Forming Test Substances.

A series of channel-forming and conductance-altering substances have been utilized in testing the patch microprobe system with membranes composed of different phospholipids. The majority of these substances are available from Sigma Chemical Co. (St. Louis, MO): gramacidin [Sigma G-5002 from Bacillus brevis (Dubos)], gramacidin S [G-5127 from Bacillus brevis (Nagano)], amphotericin B (A-4888 from stetomyces), gentamicin SO4 (G-3632), nonactin (N-3881 from stretomyces griseus), valinomycin (V-0627), and vancomycin HCl (V-2002). Alamethicin was a gift from Upjohn Pharmaceuticals.

## 3. METHODS

### 3.1 Planar Phospholipid Bilayers.

The theoretical aspects and details of forming model biomembranes from monolayers of phospholipids was reported in 1985.<sup>1</sup> General principles of the electrophysiological methods employed in measuring transmembrane electrical phenomena in the macroprobe systems [e.g., black lipid membranes (BLM)] have also been discussed. The information in the previous report just noted may be extrapolated directly to the present investigation. In this study, a microprobe method has been developed in which the planar lipid bilayer is formed across the 1- to 2- $\mu$ m opening at the tip of the patch micropipette rather than across the 0.7-mm aperture in the Teflon film septum of a large BLM recording chamber. A gigohm seal between the glass micropipette and the lipid film creates an isolated, sturdy patch of membrane suitable for voltage clamping and the investigation of transmembrane conductance changes. In addition, this microprobe system requires less material for membrane manufacture and "receptor" analysis, eliminating the need for obsessive cleaning of the larger chambered BLM system, and offers greater experimental control to the investigator. Finally, the patch micropipette system conforms to a "probe" design inasmuch as the membrane is strong enough to withstand a flow-cell environment or a dipping into multiple-cell sample trays. Because of unique conceptual and pragmatic considerations, the name patch microprobe system has been adopted to describe and to differentiate this methodology.

### 3.2 Manufacture of Patch Micropipettes.

In search of a simple, reproducible, and inexpensive micropipette manufacturing method, a wide variety of glass capillary tubing was tested, and a number of pipette pulling and microforging methods were tried. Table 1 presents a list of glass capillary tubing tested, including an evaluation of manufacturing cost, difficulty, and suitability for the project.

The majority of these glasses were tested on a Kopf 700C puller modified for patch micropipette manufacturing. This involved a set procedure of triple pulls to produce a short tank

pipette with a distal opening of approximately 2  $\mu$ m. Microforging was performed on a custom-built forge. Suitability was a judgment based on the success rate of forming stable gigohm-seal patch membranes across the pipette in 250 mM KCl and holding potentials ranging from -20 to +50 from lecithin: sphingomyelin standards (5:1), egg lecithin: phosphatidic acid (4:1), or phosphatidylcholine: cholesterol (4:1 or 5:1). Retesting of the more favorable types of glass was done later on a Sutter P-30 puller system with and without microforging. The inexpensive Fisher Scientific Blue-Tip capillary tubing achieved high performance at low cost with no microforging after a double pull. This glass type has been adopted for all subsequent studies.

Table 1. Comparison of Different Glass Capillary Tubing for the Manufacture of Patch-Type Micropipettes/Microelectrodes

<u>Source</u>	<u>Catalog No.</u>	<u>Cost</u>	<u>Microforging</u>	<u>Suitability</u>
A-M Systems	6010	+++	Yes	+/-
A-M Systems	6035	+++	Yes	+/-
A-M Systems	6175	+++	Yes	-
Clark EMI	GC150TF-15*	++++	No	+++
Drummond	100	++	Yes	-
Fisher	02-668-40	++	Yes	++
Fisher	02-668-68	+	No	+++
F. Haer & Co.	30-30-0	+++	Yes	+/-
Kimax	34505*	+	Yes	+
Mertex	MX-999	+	Yes	+/-
WPI	TST150	++++	Yes	++
Yankee	1020*	+	Yes	+/-

\*Must be acid-washed for best results.

+/- Means questionable suitability because of low success yield in trials and/or difficulty in microforging.

Some investigators follow an involved acid-washing, acetone-rinsing ritual to assure ultraclean glass prior to pulling micropipettes. We compared acid washing in some cases and found, without question, that consistently (90%) clean-tipped pipettes were drawn ready for microforging in comparison with about a 50% success rate with most nonacid-washed, acetone-rinsed capillary tubing. Acid washing is not worth the effort with the inexpensive Fisher Blue-Tip tubing if one is willing to pull



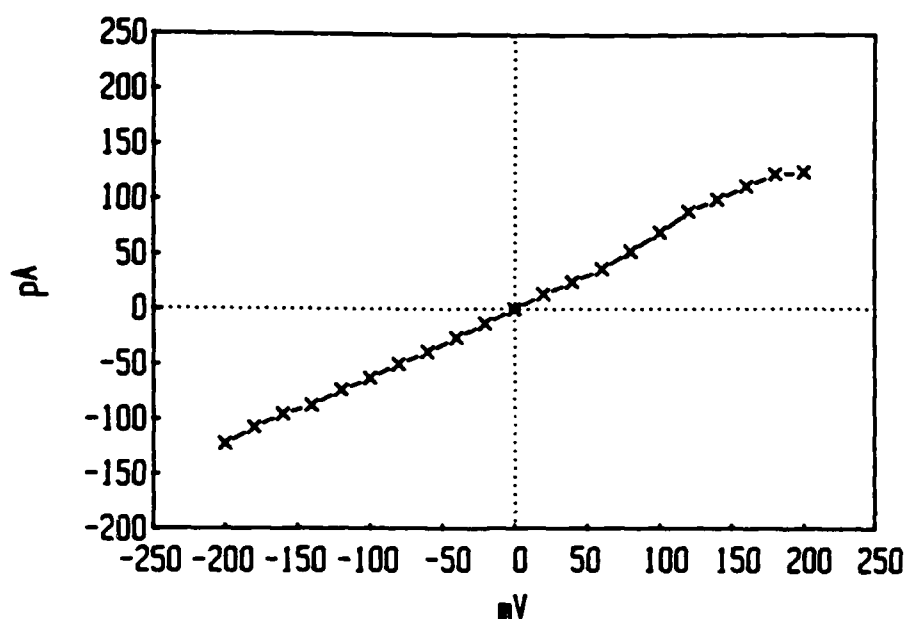
extra pipettes to offset those discarded. It was found that coating the glass with sylgard or silanizing was of no benefit in either seal formation or in improving the signal-to-noise ratio. Lastly, micropipettes cannot be reused; once a seal is formed in association with lipid material and broken, resealing in an attempt to manufacture a new membrane is not trustworthy. Thus, micropipettes are discarded after a single use.

The best settings on the Sutter P-30 micropipette puller are determined to be: first pull 8 mm in 160 arbitrary heater setting; reset to 4 mm; second pull at 103 arbitrary heater setting, no trip flag; pull strength/solenoid trip setting at 150 arbitrary units on digital readout. A tungsten or platinum coil heats under constant current controlled by the arbitrary numerical setting in order to melt the glass capillary tube. Two of three separate pulls of the molten glass (the first one or two without pulling the glass apart) are required to shorten the shank for a better signal-to-noise ratio and easier seal formation.

The micropipette can be further refined at this point by microforging. A procedure has been adopted for rapidly filling the micropipettes with electrolyte solution. Micropipettes are filled with ultrafiltered ( $0.2\ \mu\text{m}$ ) 250-mM KCl solution by immersion of the tip for 60 s, then backfilled in the base/shank portion with the same electrolyte solution with the aid of a 30-gauge needle. Drug or other ions can be added at this stage. The filled micropipettes are slipped over an AgCl pellet attached by Teflon-coated Ag wire to an acrylic holder. The holder is then connected to the headstage socket mounted firmly in a vertical micromanipulator.

The micropipette is driven into the medium (e.g., 250 mM KCl) of a recording vessel, then noise (RMS  $<3.0\ \text{pA}$  on AxoPatch digital readout) and resistance (i.e., impedance; approximately  $10\ \text{M}\Omega$ ) are measured with the aid of a calibrating pulse from the AxoPatch amplifier to ascertain the quality of the filled micropipette-microelectrode. Micropipettes not meeting these criteria are discarded.

Microforging is a technique developed to modify the tips of micropipettes. A correctly shaped tip may be critical for achieving the gigohm seal in patch clamping. A smoothly "fire"-polished surface at the tip of a pulled capillary tube is not easily accomplished during pulling and separating the molten glass. Thus, thermal polishing of the tip is required (Figure 5). The double- or triple-pulled micropipette is clamped in a horizontal micromanipulator in plane with the stage of a light microscope and viewed initially at 40X. The point of a fine, looped filament of platinum/iridium (Pt/Ir) is driven close to the tip of the micropipette via another horizontal micromanipulator.



Curve is generated by manually stepping at 10-mV intervals and recording the pA value for each step. The symmetry of this curve suggests a symmetrical bilayer structure in which gramacidin is unbiased by the polarity of the voltage. In other words, gramacidin appears to distribute to both sides of the bilayer, operating without a single-sided voltage preference.

Figure 5. Current-Voltage Curve for Gramacidin in a Lecithin-Sphingomyelin Patch Bilayer

The microscope is switched to approximately 600X. Low current is passed through the Pt/Ir filament under the control of a rheostat. Care must be exercised that the expanding filament does not contact the glass. Close observation of the micropipette's tip will reveal a visible change of refractive index of the glass, which signals local melting forging of the tip. The current is then terminated. Using this method, acceptable micropipettes can be consistently reproduced without the need for expensive, commercially available timing and heat-sensing circuits.

Only pulled micropipettes with a uniformly shaped tip should be forged. Irregularly shaped or cracked tips cannot be forged into a suitable shape for patch formation. Forging produces a smooth, rounded surface for the lipid seal. Hard glass requires forging; the softer Fisher Blue-Tip tubing apparently melts to a relatively smooth tip during the triple pull and does not require forging.

Several technical aspects of the heater filament of the microforge are noteworthy. Either Pt/Ir or "Nichrome" wire (0.2-0.4 mm) may be used. A 2-cm piece of wire is bent in two to form a sharp point (hairpin shape) at the center. The two ends are secured in a clamp and soldered to lead wires from the rheostat and transformer. The wire is carefully coated with glass while red hot, using thin filaments of drawn Pasteur pipettes.

Only the tip remains bare. This minimizes vaporization of the metal and aids in maintaining a constant tip temperature during heating. Vapor deposits from this process may damage the microscope objective lens over time. An industrial grade lens may be purchased or a standard lens can be protected with replaceable coverslip glass. Overheating the filament, even briefly, will destroy it.

### 3.3 Methods of Forming an Artificial Biomembrane Patch.

Formation of a planar phospholipid bilayer across the tip of a properly prepared patch-type micropipette is straightforward in concept, but more difficult in practice. Once a low-noise, stable micropipette has been mounted in the electrode holder, the micromanipulator is used to vertically drive the micropipette 1 to 2 mm below the surface of the appropriate physiological medium in the recording chamber. A phospholipid monolayer is then spread over the surface by applying a mixture of phospholipids (1-3  $\mu$ L) in a volatile solvent such as pentane, hexane, or decane (10-50 mg/mL) to the edge of the chamber. Incident bright light will reveal a faint shadow spreading over the surface if the application is successful.

Sonicated lipid-protein mixtures can also be applied in this manner, although monolayer formation may take 10 to 15 min, therefore, raising concern for oxidation of the lipids. Then 5-15 min is allowed for the evaporation of the solvent from the lipid-air interface, the lighter chain hydrocarbons taking less time. One must be cautious in allowing longer chain hydrocarbons (such as decane or above) to evaporate because they may form transient pseudomembrane structures. With the micromanipulator, the micropipette is slowly, then rapidly raised and lowered so that the tip passes through the phospholipid monolayer five or six times or more.

While conducting this procedure, the  $R_{\text{seal}}$  Test module of the AxoPatch is monitored using a 10-mV oscillation at 10 Hz. A distinct diminution of the pulses in an RC-filtered manner signals seal formation. Using the relationship  $G\Omega = \text{mV/pA}$ , the gigohm magnitude of the seal (usually 3-5 or more) is determined from the current-output monitor. About one in every three attempts with a new micropipette results in a good, stable gigohm seal. A holding potential of +20 mV is usually applied to the patch of membrane. Voltage differentials of >150 mV applied to the membrane at command steps may break the membrane, depending on the lipid composition. Voltage gradients across the membrane mimic mammalian cells in vivo and provide the environment for transmembrane conductance changes via ionic channels.

The membrane is voltage clamped (or "patch" clamped since only a minute patch of membrane is under study), and the resulting current fluctuations required of the clamping amplifier to hold the membrane voltage steady reflect channel openings and

closings and other alterations in conductance (receptor-mediated, artificial pore formation, phospholipid disintegration, etc.). Thus, this methodology is ideal for examining electrical signal transduction across biomembrane structures and forms the basis of the patch microprobe detection scheme.

### 3.4 Reconstitution Trials with Muscle Homogenates and Sonicates.

A preliminary study was designed to test the developing patch microprobe system on extracts from mammalian tissue. In addition, earlier interest in the study and sources of chloride channels provoked an assessment of the potential of using crude extracts of homogenates of smooth muscle tissue (rat ileum), skeletal muscle (rat hemidiaphragm), and rodent erythrocytes. Tissues were dissected from rats killed by cervical dislocation; blood was collected by intracardiac puncture. A polytron homogenizer was used to rapidly homogenize the tissue (approximately 30 mg) in 100 mM-sodium phosphate buffer adjusted to pH 7.4. A chloroform:methanol extraction (with acetone to clear the neutral lipids) was used to collect phospholipids for membrane formulation.

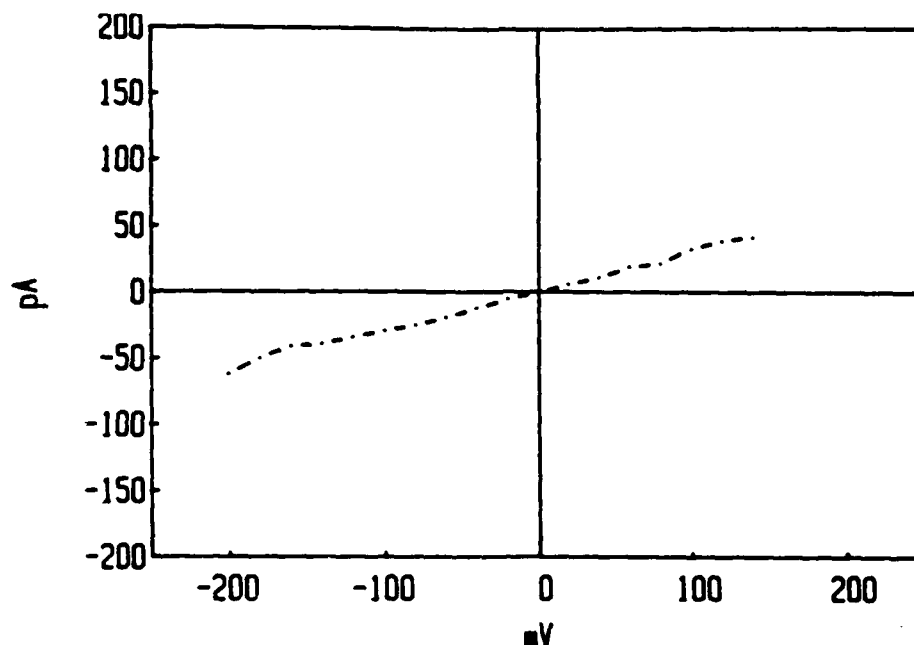
Aliquots of this mixture were dried under nitrogen and redissolved in n-hexane. Gigaohm seals were readily obtained across the patch micropipette with these lipid extracts. These membranes gave more noise than bilayers of pure phospholipid combinations and were subject to intermittent jumps in conductance and disintegration. Aliquots of protein-containing aqueous extracts were sonicated in n-hexane to produce liposome structures. These were applied to the chambers without success in an attempt to form monolayers for patch formation.

Undefinable channel-like membrane-conductance fluctuations occurred in two experiments when the liposomes were introduced into the medium (trans-side) of a preformed membrane (phosphatidyl choline:sphingomyelin:cholesterol, 10:2:1). Reexamination of this approach evoked a rejection of this crude method until defined receptors or channels of interest could be identified and characterized in a true on-cell, patch-clamping situation, perhaps in cultured cells [Redelman, (University of Nevada, Reno, NV) personal communication]. Nonetheless, this method will likely succeed in reconstituting isolated enzyme fractions such as  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -ATPase.

### 3.5 Channel-Forming Test Substances.

Current-voltage (I-V) curves were determined on gramicidin, nonactin, and alamethacin (Figures 6-8). These substances were chosen as representatives of each characteristic subgroup: spontaneous channel-former, carrier, and voltage-dependent channel-former. The protocol determined the curve by manually stepping the voltage of a tight-seal membrane with agent through

+200 mV in 10-mV steps. Future experiments will be performed using the pCLAMP D/A output to voltage ramp at a smooth frequency of 0.5 Hz over the test range.



This curve is generated by recording current flow (pA) while manually stepping through a -200 to +150 mV range in voltage steps of 10 mV. In this experiment, the membrane disintegrated due to large conductance changes above 150 mV. The symmetry of this curve suggests uniformity in the distribution and activity of the compound and the phospholipid bilayer structure.

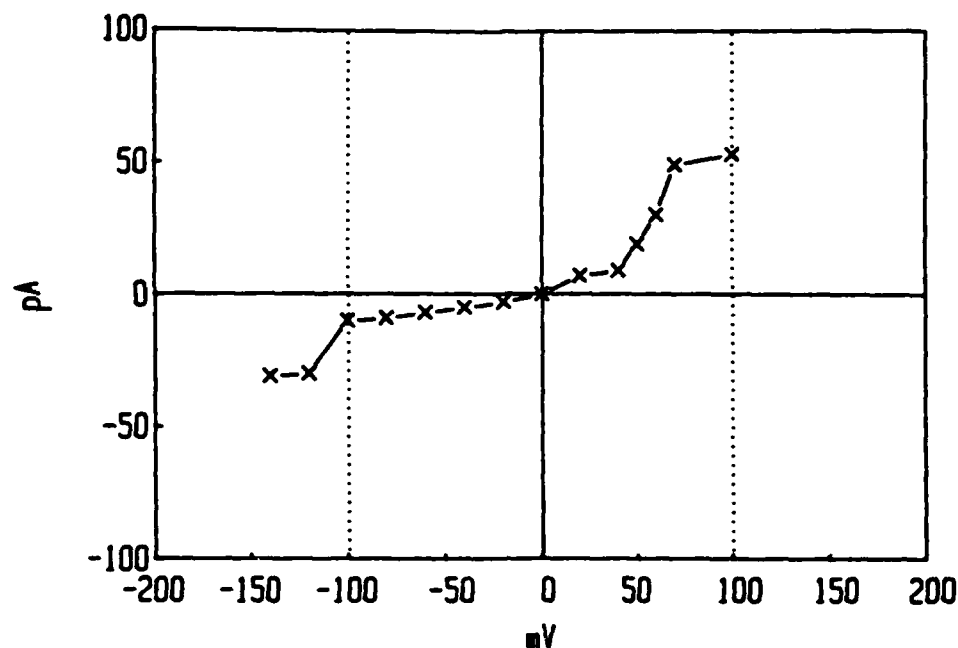
Figure 6. Current-Voltage Curve for Nonactin in a Sphingomyelin Patch Membrane

### 3.6 Cholinergic Drug Trials.

Diisopropylfluorophosphate (DFP) in anhydrous propylene glycol stock ( $10^{-2}$  M) and d-tubocurarine (dTC) were examined in gramicidin-seeded lecithin:sphingomyelin (5:1) patch membrane. DFP induces disruption of BLM membranes in the macroprobe system; consequently, it was of interest to determine if DFP had a deleterious effect on the more sturdy patch membrane. Two microliters of propylene glycol were applied to the 0.7-mL-volume center well of the recording chamber to test the vehicle. No change in conductance was noted, although a full I-V curve was not plotted.

DFP stock in propylene glycol (2  $\mu$ L) had a slight effect, inducing greater leakage current and lowering the blowout threshold from +200-250 mV to 176-189 mV ( $n = 2$ ). Gramicidin channel-like activity did not appear to change dramatically with DFP. Occasional prolonged conductance jumps occurred with

gramacidin activity superimposed. The pCLAMP analysis is ideal for dissecting modifications in channel-like activity in this situation and will be used in future studies.

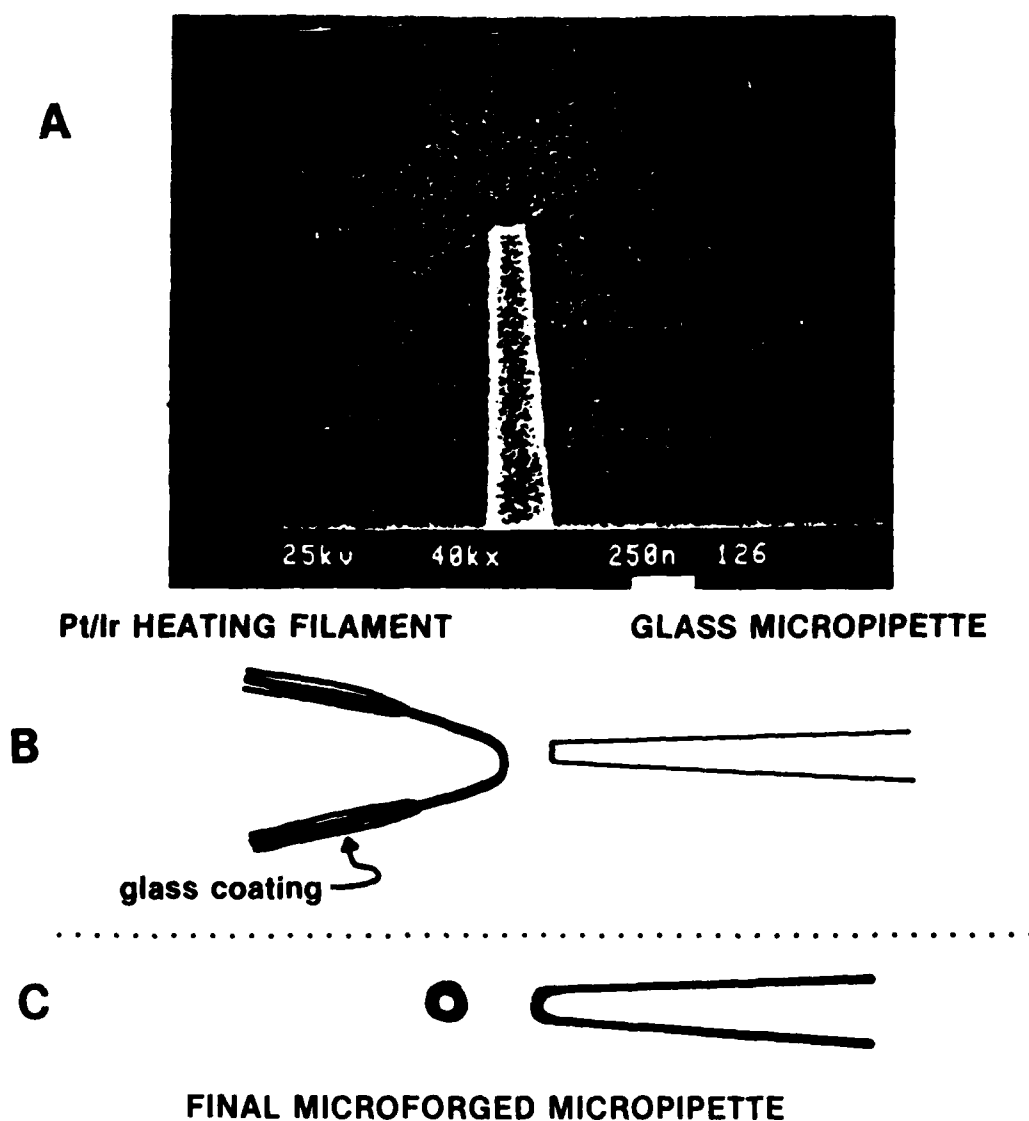


Unlike Gramacidin and nonactin, alamethicin is a voltage-dependent "channel" former which behaves in an asymmetric manner when introduced to only one side of the phospholipid bilayer. In this experiment alamethicin was introduced on the cis (intrapipette) side of the bilayer. Note the steep I-V curve on the positive voltage ramp, but the sluggish current flow on the negative voltage-clamping ramp.

Figure 7. Current-Voltage (I-V) Curve for Alamethacin in an Artificial Patch Biomembrane

### 3.7 Sources and Study of Anion Channels.

Several threat agents are postulated to act on chloride channels. Consideration was thus given to finding a convenient source of chloride channels for study. Like other ionic channels in excitable tissue, chloride channels may or may not be coupled to a neurotransmitter receptor (e.g., GABA receptor). Hille<sup>2</sup> divides Cl channels into three categories: steeply voltage-dependent channels, weakly voltage-dependent "background" channels, and transmitter-operated synaptic channels. Chloride channels are viewed generally as "opposing followers" linked to cationic channels; thus, they would oppose normal excitability, help to repolarize a depolarized cell, play a role in the regulation of intracellular pH, and regulate intracellular volume. The "background" chloride channels were not considered a fruitful area to begin studies using a patch membrane. Nonetheless, myotonic disorders stress the importance of these "passive" channels in excitable tissue.<sup>3,4</sup>



Panel A is a scanning electron micrograph of a micropipette pulled on the Sutter P-30 puller. Note the unrounded shape of the tip.

Panel B shows the microforging method for altering the roughly snapped tip of the double pulled pipette.

Panel C shows the ideal smooth, rounded shape of the finished patch micropipette. The glass at the tip is slightly thickened by this process; however, if too thick it will occlude the opening. A slightly thickened tip with a smooth fire-polished surface provides an ideal glass-lipid interface suitable for forming a stable, long-lasting gigaohm seal.

Figure 8. Microforged Micropipette

Common muscle preparations were examined for nonneurotransmitter-linked chloride channel sources, but with the requirement of measureable voltage-dependence. Distinctly voltage-dependent chloride channels have recently been discovered primarily in lipid bilayer work as part of reconstitution studies. In almost all the studies, chloride channels were not the focus of the experiments. Chloride channel conductances appear to be much lower than equivalent cation channels and have

been detected in bilayers with fractions from Torpedo electrical organ,<sup>5</sup> heart membrane vesicles,<sup>6,7</sup> cultured rat myotubes,<sup>8,9</sup> and *Aplysia* neurons.<sup>10</sup> Therefore, it appeared that a reasonable approach to gain access to a definable, voltage-dependent chloride channel was to attempt the reconstitution of extracts from skeletal and smooth muscles.

### 3.8 Activity of Acetylcholinesterase on Silicon Monoxide Substrates.

Six 12.5-mm<sup>2</sup> silica wafers covered with silicon monoxide were washed in chloroform:methanol (2:1) and then in acetone. Each wafer was placed in a small (7-mL) plastic scintillation vial (Beckman 865253) under 20% 3-aminopropyltriethoxysilane (Sigma No. A-3648) in anhydrous toluene. Two milliliters of this silanizing mixture were sufficient to cover the wafer, which remained upright in the vial. Silanizing mixture may be conserved by placing two wafers in each vial. The vials were mildly shaken for 2 hr on a mechanical shaker at room temperature. The organic solvent was removed by a drawn Pasteur pipette connected to a vacuum trap source. The wafers were washed five times with toluene, then dried under a stream of nitrogen in a fume hood. It is presumed that the 3-aminopropyltriethoxysilane produces a surface of reactive amino groups on reaction with Si-OH groups of the silicon monoxide surface. Conditions would theoretically exist for peptide bond formation with the crude enzyme preparation.

Acetylcholinesterase (EC 3.1.1.7; Sigma Type V-S from electric eel; C2888; 200 units) in buffered salts was reconstituted with 980  $\mu$ L of double-glass-distilled water. At pH 8.0 and 37 °C, one unit of activity will hydrolyze 1.0  $\mu$ mol/min of acetylcholine to choline and acetate. Fifty microliters of this mixture was applied to each 12.5 mm<sup>2</sup> silica wafer and spread over the substrate surface. Surface tension was generally unfavorable for even spreading. In later experiments, two wafers were used to form a sandwich with the enzyme mixture in between. After 1 hr, the wafers were washed gently with sodium phosphate buffer, 100 mM, pH 7.4. The enzyme-coated wafers were then transferred to the reaction vial to test enzymatic activity.

A modified, sensitive acetylcholinesterase radioenzymatic assay<sup>11</sup> was used to test the enzyme's capability to hydrolyze 14C-labeled ACh. The buffer substrate consisted of 2 mL of 100 mM sodium phosphate buffer, pH 7.0, and 100  $\mu$ L of 1-14C-ACh chloride, 100 mM (20 uCi/mM) stock; 2.0 mL total. Small plastic scintillation vials (7 mL) were used as the reaction vessels because the flat bottom reduces the amount of buffer substrate required. Three glass beads, each 3 mm in diameter, were placed on the non-silicon monoxide substrate surface to further reduce volume. The reaction mixture consisted of 200  $\mu$ L of buffer substrate and 400  $\mu$ L of sodium phosphate buffer, 100 mM, pH 7.4. The protocol consisted of placing the wafer and the glass beads into the vial,



introducing the reagent and incubating for 30 min at 30 °C. The reaction was stopped by adding a mixture of 400 µL of sodium phosphate buffer (10 mM, pH 6.6) and 1.5 mL of tetraphenylboron in 3-heptanone (75 mg/mL). The liquid was drawn off by Pasteur pipette to a glass culture tube (10 x 75 mm) and mixed vigorously with a vortex mixer. The mixture was centrifuged at low speed (4 °C) to assure phase separation. This mixture traps the unreacted ACh in the tetraphenylboron; the <sup>14</sup>C-acetate remains in the aqueous phase. The top organic phase was aspirated and discarded. An aliquot (400 µL) of the aqueous phase was added to 5-mL scintillation cocktail (Beckman Ready Solv MP) in a small plastic counting vial. The vials were counted on a Packard Tricarb 460D scintillation counter for two runs at 20 min/vial.

A comparison of counts/min (cpm) from blank vials (only scintillation cocktail); vials with silanized, nonenzyme-treated "plain" wafers; and vials containing treated wafers was made to determine the presence of <sup>14</sup>C-acetate, which would indicate hydrolysis of ACh. Aliquots of buffer substrate and the buffer substrate mixture were also counted for each experiment to ascertain the radioactivity available to the acetylcholinesterase. In two experiments (n = 2) the activity of 50 µL of reconstituted enzyme (no wafer) was tested against the buffer substrate mixture (600 µL). This established that the reconstituted enzyme is highly active, hydrolyzing 99.9+% of the <sup>14</sup>C-ACh in 30 min at 30 °C.

Five wafers were washed and silanized as described above. Four of these wafers were treated with reconstituted AChE; one served as control. AChE assays were done on all five wafers. Conditions were unvaried in the three separate experiments.

Two experiments were performed to examine the pH optimum. For each experiment, 12 wafers were prepared as described above. New radio-labelled ACh was obtained from New England Nuclear (NEC-3350). The reaction mixtures were adjusted to pH 6.0, 6.6, 7.0, 7.4, or 8.0. Two wafers were run at each pH point, with two nonenzyme, silanized wafers serving as control blanks.

#### 4. RESULTS

##### 4.1 Lipid Compositions.

A variety of lipids found in natural excitable biomembranes have been tested alone or in combination to determine their suitability in forming artificial membrane patches. This is largely new territory, because few investigators have explored the formation of artificial biomembrane on patch micropipettes for the purpose of developing detection devices.

The primary interest has been in how easily the lipids form a membrane, how stable the resulting membrane is, how long

the membrane lasts, and whether the membrane will accept channel-forming molecules or protein moieties. Attention has been paid to selecting combinations of phospholipids which affect the bilayer environment respective of inserting other molecular species [e.g., PE and cardiolipin form a charged bilayer, PE/PC are neutral; and PC/PS form an asymmetrical bilayer on glass but not on Teflon, which was frequently used by earlier workers using BLM (Coronado and Latorre, 1983)].<sup>12</sup> It was discovered that some phospholipids form membranes readily but break up too quickly or are too voltage unstable for experimentation. Other phospholipids were difficult to form, but once formed, were very stable. Table 2 presents a list of the lipids tested and rates each one or combination on a number of parameters.

Most lipids are received or are prepared in a stock solution of chloroform, methanol, or both. Aliquots of these lipids are dried under a stream of nitrogen just prior to the experiment and redissolved in hexane, pentane, or decane. Several microliters of this stock are used to form the bilayer. Since the solvent may influence the characteristics of the bilayer, it is identified in Table 2. Ease of seal formation is rated subjectively as very easy (++), average (+), or difficult (-). Temporal stability rates the duration of the seal once formed and is given in a range of minutes where measurements were made; this only serves for comparison purposes. Insertion potential estimates the utility of the lipid or combination for future reconstitution or receptor-insertion projects based, in part, on the behavior of channel-formers such as gramicidin.

Several observations, some unexpected, were derived from studying the seal-forming ability and stability of the various lipids. Cholesterol did not significantly increase the stability of the patch membranes and, in fact, subjective observations suggest that 10-20% cholesterol may actually destabilize the membrane at 18-22 °C. Patch membranes containing cholesterol were less tolerant of high voltage steps and often blew out prematurely. This observation differs from evidence with a larger membrane (BLM) which appears to be stabilized by small percentages of cholesterol or have lower leakage currents.<sup>12,13</sup> This could be a function of the type of glass used or the unique environment (e.g., surface tension) of the patch micropipette. In addition, it could result from the concentration of cholesterol used in the present experiments as compared with the 20:1 ranges of phospholipid:cholesterol ratios used by other investigators.

Membrane stability appeared to decrease with most phospholipids at the high and low temperature ranges tested early in the study. For instance, membranes formed with the favored standard mixture of lecithin:sphingomyelin progressively disrupted at 32-40 °C and showed inconsistent channel activity with gramicidin at <10 °C. In several trials with other lipid compositions, it became apparent that developing a system for room

Table 2. Tests of Potential Patch Membrane-Forming Lipids

Lipid	Source	Solvent	Ease of Seal	Temporal Stability	Insertion Potential
Diphytanoyl-lecithin*	Avanit	Hexane	+/-	90-130	Very good
Phosphatidic acid*	Sigma	Hexane	++	60-90	Good
PA:lecithin 4:1	Sigma	Pentane	+++	>90	Good
Cholesterol	Sigma	Hexane	--	----	None
P'tdyl ethanolamine	Sigma	Pentane	+	30-60	Mixed
P'tdyl serine	Sigma	Pentane	+	<60	Mixed
P'tdyl choline	Sigma	Hexane	++	45-70	Fair
Cardiolipin	Sigma	Pentane	+/-	<30	?
P'tdyl inositol	Sigma	Pentane	++	40-50	Mixed
PC dipalmitoyl	Sigma	Hexane	++	<60	Mixed
PC dilauroyl	Sigma	Pentane	+	<60	Mixed
L:S* 5:1	Sigma	Pentane	+++	>60	Good
L:S 1:1	Sigma	Pentane	+	>60	Fair
L:Chol 4:1	Sigma	Pentane	+/-	60-90	Good
L:Chol 5:1	Sigma	Pentane	+	>60	Good
L:Chol 4:1	Sigma	Decane	++	<60	?
PS:Chol 4:1	Sigma	Pentane	+	<30	Good
PS:PC:Chol* 2:2:1	Sigma	Hexane	++	>60	Good
PS:PC:Chol 1:1:1	Sigma	Hexane	++	60-90	Good
PE:Chol 4:1	Sigma	Hexane	+/-	<60	Fair
PS:PC 1:1	Sigma	Pentane	++	60-90	Fair
Cardiolipin:lecithin 1:1	Sigma	Pentane	+/-	<30	Poor

Legend: Lipids were applied to chamber in a concentration of 5-15 mg/ml of solvent.

P'tdyl, phosphatidyl; PA, phosphatidic acid; PC, phosphatidyl choline; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; L:S, lecithin:sphingomyelin standards received in fixed ratios in chloroform:methanol (1:1); L:Chol, lecithin:cholesterol.

Mixed indicates better performance mixed with another phospholipid and/or with cholesterol (10-20%).

Addition of alpha-tocopherol (0.5%) to mixtures of PC, PS, or CE prolonged the integrity of the membrane and appear to reduce the noise levels which often increase as the membrane ages signalling impending breakdown and loss of the gigaohm seal.

All phospholipid used were of the L-alpha configuration.

temperature (18-22 °C) would be more appropriate. That is, membrane stability significantly decreases at higher temperature, perhaps by enhanced autooxidation and increased fluidity. Furthermore, maintaining the membrane-micropipette-recording chamber at closely controlled temperatures between 30-40 °C is difficult when changing solutions; slight fluxes in temperature will alter the behavior of the membrane. Moreover, working at decreased temperatures (4-10 °C) quickly revealed serious difficulties inasmuch as the basic behavior of phospholipids (and cholesterol) drastically changes below the transition temperatures of the individual lipids. Seals were very difficult or impossible to form at <10 °C, even with mixtures of polyunsaturated phospholipids. It is uncertain whether gramicidin penetrated the several membranes we were able to form, since discrete channel-like activity was not detected.

Another observation of value is our preference for lipids of soybean or synthetic sources, rather than from egg or animal sources. This is due to the different compositions of the fatty acid chains on the phospholipid backbones. Lecithin of soy origin was more stable than lecithin from eggs. L-alpha-diphytanoyl-lecithin (a synthetic from Avanti) proved to be the most stable and favored phospholipid of the entire study. It is very stable; resists oxidation; forms sturdy, long-lasting membranes; and accepts any of the channel-forming antibiotic molecules tried. It is intended to use this lipid, although expensive, in future receptor insertion studies. An additional mixture of marked interest is the combination of lecithin (>90% phosphatidyl-choline):sphingomyelin (5:1) applied in pentane or hexane. This balanced mixture readily formed long-lasting patch membranes that accepted channel-forming molecules. In one experiment, a stable membrane lasted for 3 hr. These membranes were also resistant to blowout voltage steps. Thus, this mixture would be favored in receptor reconstitution studies.

Finally, some mixtures (e.g., soy lecithin 10 mg/0.7 mL hexane) were sonicated prior to use in order to produce liposomes or micelles in early trials to mimic reconstitution methods. Membrane formation occurs after long delays once the lipid is added to the surface of the chamber. We suspect that the liposomes slowly break up and form a monolayer which then yields to the membrane manufacturing procedure of moving the micropipette through the monolayer surface numerous times.

#### 4.2 Channel-Forming Test Substances.

In general, gramicidin, gramicidin S, amphotericin B, gentamicin SO<sub>4</sub>, nonactin, valinomycin, and vancomycin have slight to moderate solubility in aqueous solution; they actively induce conductance alterations in concentrations of approximately  $10^{-9}$  M. When dissolved in the electrolytic medium filling the

micropipette, these compounds can be introduced into the patch membrane that forms across the tip. The two sides of the artificial planar phospholipid membrane are often designated as cis and trans to distinguish the environment inside the pipette versus the bath medium for the purpose of clarifying the site of drug application or change in ionic gradient. Introduction of the drug to the inside of the micropipette offers excellent control over concentration gradients and conserves material. Substances like gramicidin will enter the planar phospholipid bilayer and within minutes begin to induce discrete "potassium" channel activity very similar to that observed in intact cells.

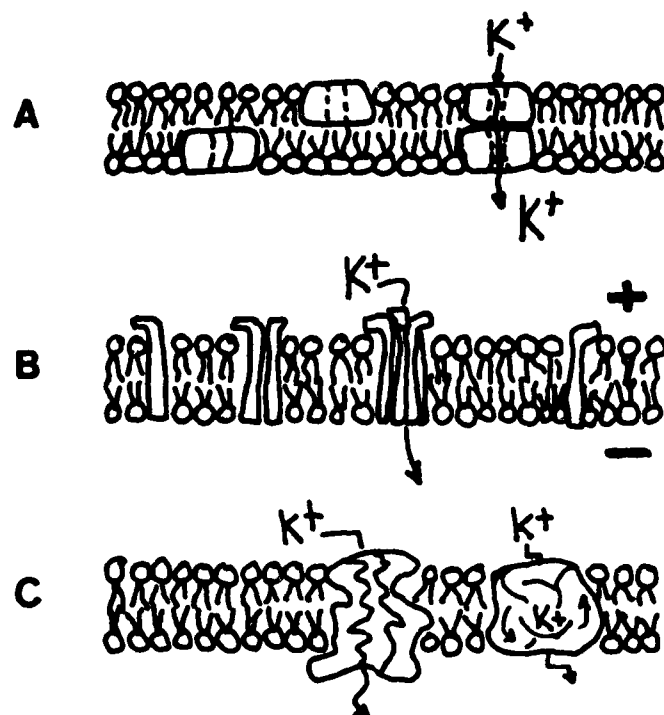
Routinely, concentrations of  $10^{-8}$  and  $10^{-9}$  are required; higher concentrations may disrupt the membrane due to dramatic fluctuations in conductance. Other molecules in the group appear to act as ion carriers in lipid bilayer membranes. Valinomycin and nonactin (a macrotetralide actin) enter the bilayer, decrease the resistance, and induce large conductance changes by creating a mechanism to carry large currents of cations across the membrane. Alamethacin induces channel-like conductance changes in the artificial membranes dependent on the magnitude and polarity of the voltage applied. It is, therefore, a voltage-dependent channel-former and exhibits asymmetrical behavior if placed on only one side of the membrane or in an asymmetrical lipid bilayer.

Not all the compounds were studied extensively, yet each was determined to induce conductance fluctuations in membranes formed of various mixtures of lecithin (e.g., lecithin:sphingomyelin, 5:1) or diphytanoyl-lecithin. The most remarkable and consistently studied compound was gramicidin. This compound has unique properties that verify bilayer formation, while at the same time exhibiting potassium channel-like activity (Figure 9). No advantage was discerned in studying the other compounds in detail inasmuch as our main purpose was model development and the testing of bilayer formation and stability. Nonetheless, the observing and understanding of characteristic current fluctuations across the artificial membranes was valuable in preparation for the study of isolated receptive moieties.

In current-voltage (I-V) curves, gramicidin (Figure 5) and nonactin (Figure 6) yielded largely symmetric I-V curves by cis (intrapipette) application of the agent, whereas alamethacin gave asymmetric behavior as predicted (Figure 7).

A series of computer plots is presented to illustrate our success with the patch microprobe system, using artificial phospholipid bilayer membranes to study the discrete channel-like activity of various antibiotic substances. The remarkable capability of detecting and analyzing discrete events in the range of 1 pA of current across the membrane demonstrates the sensitivity of the methodology. Figure 10 represents the channel behavior of gramicidin A in a lecithin:sphingomyelin (5:1) bilayer clamped at 150 mV with equimolar concentrations of KCl (250 mM) on both

sides of the membrane. Gramacidin was introduced via the intrapipette medium. Note the discrete steps as the "channel" opens and closes.



Panel A illustrates the theory of gramacidin channels. It is noteworthy that, based on this theory, gramacidin will not form channels in nonbilayer lipid configurations. Two monomers must form a dimer oriented to allow the passage of monovalent cations. Single unconnected monomers are too small to penetrate the lipid bilayer. Furthermore, properly oriented dimers are too small to create a channel in multilayered phospholipid films except for the bilayer.

Panel B shows a theoretical diagram of how an applied voltage may orient three or more alamethicin monomers to form a channel.

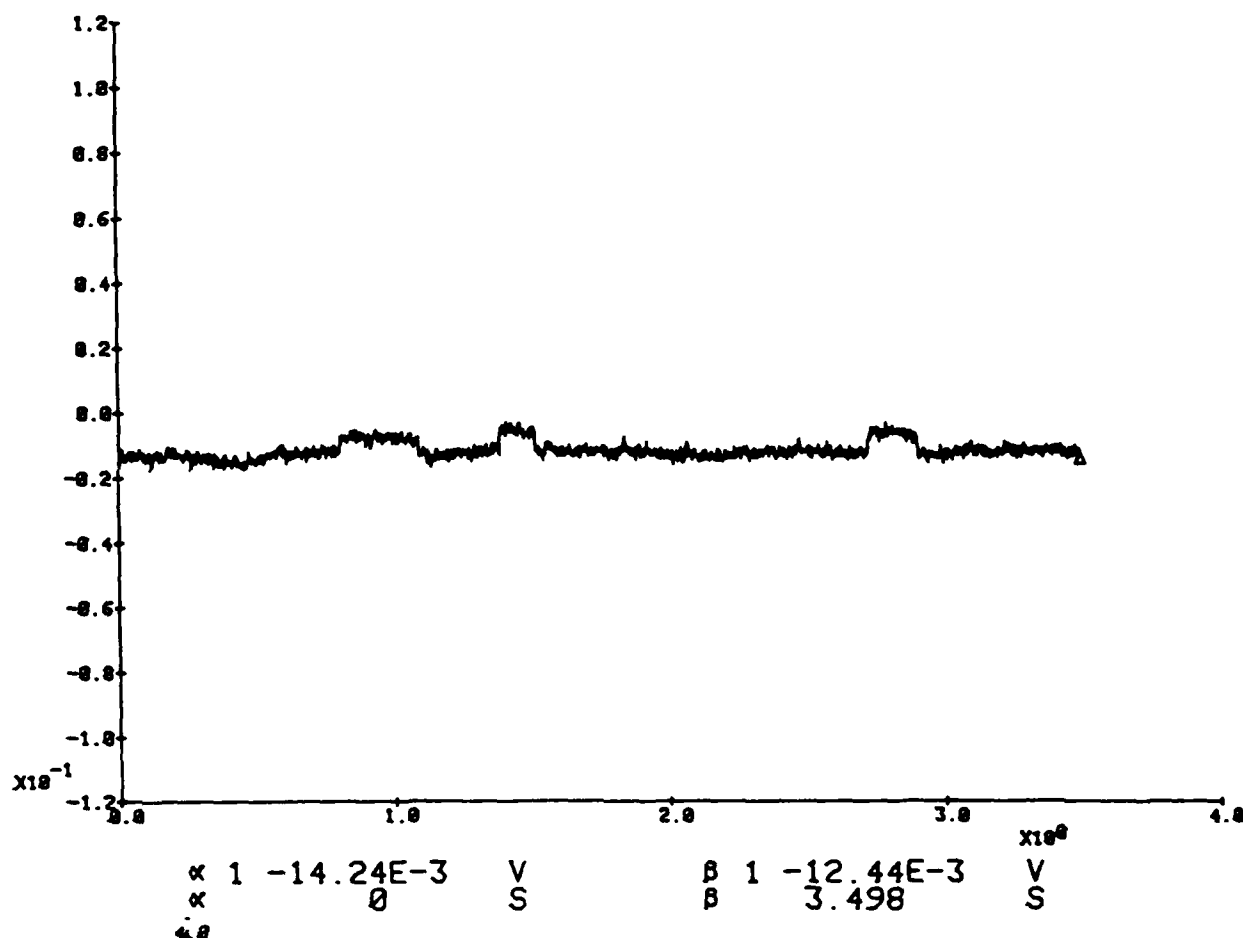
Panel C illustrates the theoretical mechanism by which ionic conductance modulators influence translocation of ions across lipid biomembranes. These may be large macromolecular structures which completely transect the membrane and form pores; or the structures may resemble valinomycin-like molecules which insert into the membrane and "carry" cations from one side of the membrane to the other either by physically rotating in the bilayer or by an internal flip-flop mechanism.

Figure 9. Diagrammatic View of How Antibiotic Molecules Form Channels in Planar Phospholipid Bilayers

Figure 11 illustrates a single-"channel" event from the same experiment. Note the excellent signal-to-noise ratio; the discrete step is approximately 0.7 pA in magnitude; the scale on the vertical axis is necessarily arbitrary since the Smartscope autoscales an AxoPatch autozeroed signal already modified by gain setting. In another experiment, the addition of d-tubocurarine (dTC,  $10^{-8}$  M) to the trans-side of the membrane resulted in transmembrane fluctuations additive to the already present gramacidin events (Figure 12). It is not known if dTC possesses

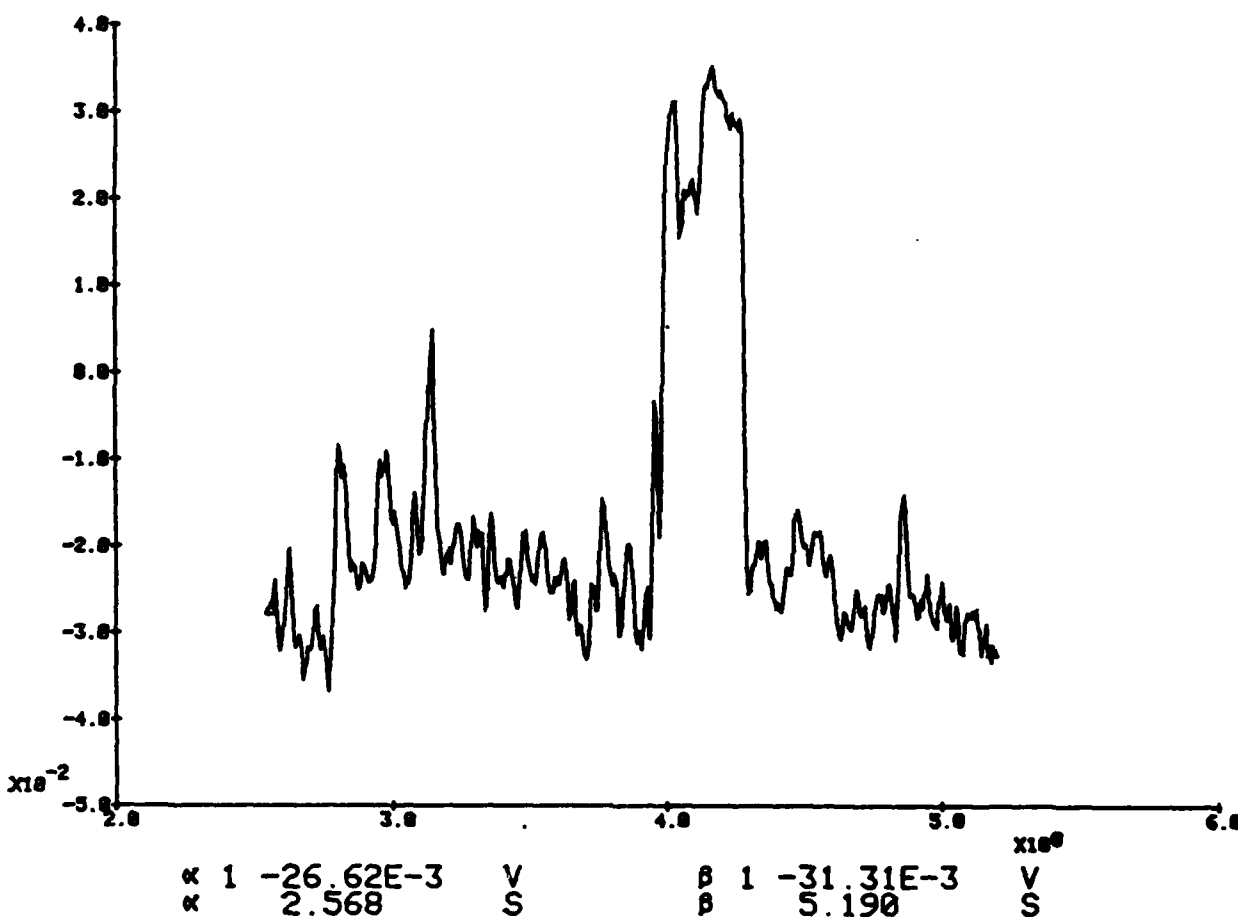
channel-like activity or whether a contaminate of the extract induced the activity. Nonetheless, the activity was not transient, nor did it affect the stability of the membrane as recordings were made for more than 60 min.

Figures 13 and 14 demonstrate the dTC events in additional experiments. Figure 13 is with gramicidin and Figure 14 is in the absence of gramicidin. These experiments show a previously unreported action of dTC in planar lipid bilayers but raises caution for experiments designed to study receptor-agonist (or antagonist) interactions.



Membrane was formed from lecithin: sphingomyelin standard (5:1) in hexane. Gramicidin ( $10^{-9}$ ) was introduced via the trans-side in the recording bath. Equimolar concentrations (250 mM) of KCl solution was used in the recording pipette and in the bath. A  $4.5 \text{ G}\Omega$  seal was achieved. The holding potential was +150 mV; a headstage gain of 1; output gain of 5;  $I = 45 \text{ pA}$ ; RMS noise level equaled 0.6 with low-pass filtering at 500 Hz. This trace was captured on the Smartscope Waveform Analyzer during an autozero run from the AxoPatch system. Discrete channel-like events occurred at random as illustrated in this 3.5-s acquisition.

Figure 10. Discrete Channel-Like Events in a Phospholipid Bilayer Induced by Gramicidin



This expanded channel-like event was captured in a 5-s sweep during the experiment described in Figure 10. These events are nearly identical to channel events described in patch-clamped biological membranes. The signal-to-noise ratio is favorable for computer analysis as shown in Figures 4 and 5.

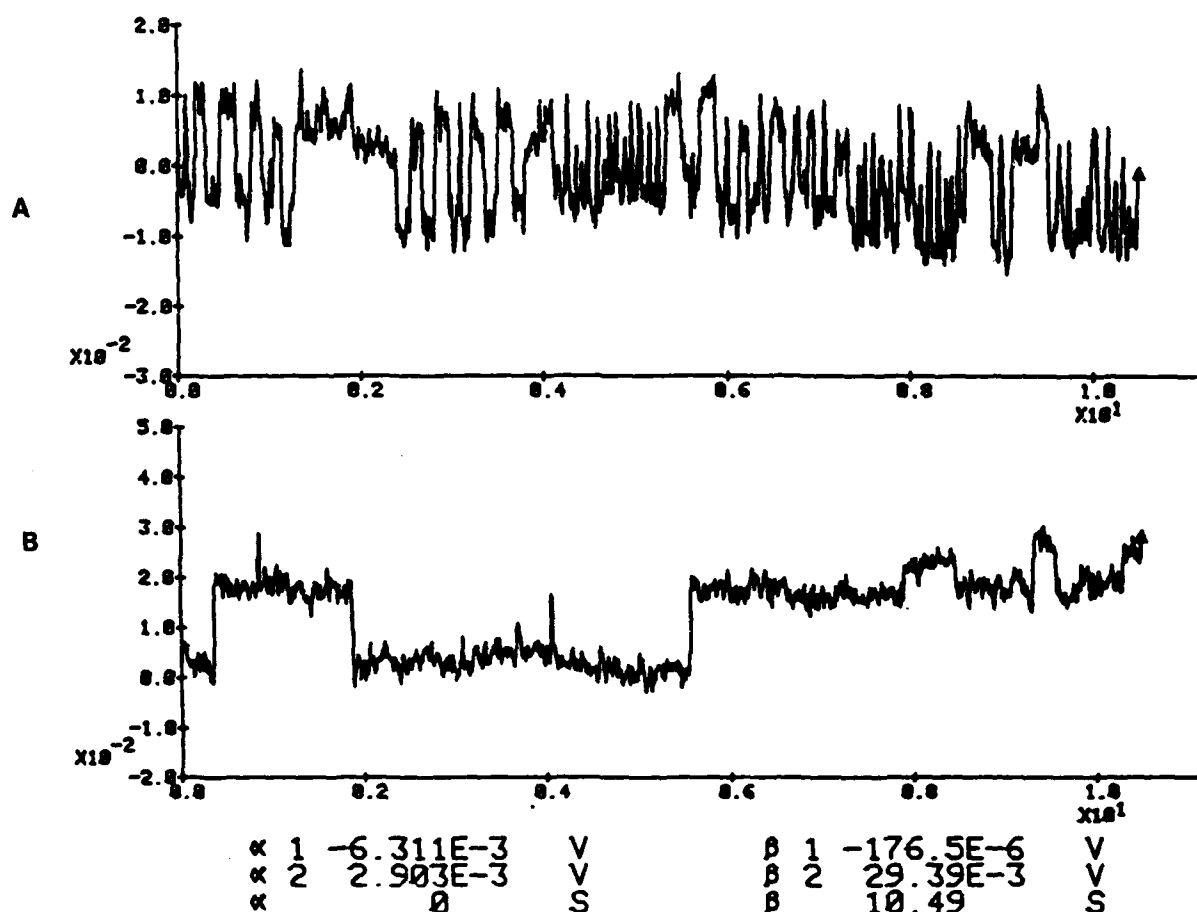
Figure 11. Single-Channel Event in a Phospholipid Bilayer Induced by Gramacidin

Figure 15 illustrates the massive conductance shifts often observed with substances like nonactin. Discrete channel-like events are not always observed. Rather, sudden large conductances occur with shifts in baseline. However, the membrane does not rupture. Other experiments with nonactin reveal conductance fluctuations approximating channel-like events, but without the precision of the gramacidin "channels." Figures 15 and 16 demonstrate the importance of selecting appropriate phospholipid mixtures for studying inserted molecules. The records shown in Figure 15 were from a lecithin:sphingomyelin (5:1) bilayer. The records in Figure 16 were from a phosphatidic acid bilayer.

Amphotericin B inserts into the bilayer and induces stepped fluctuations of longer duration than gramacidin



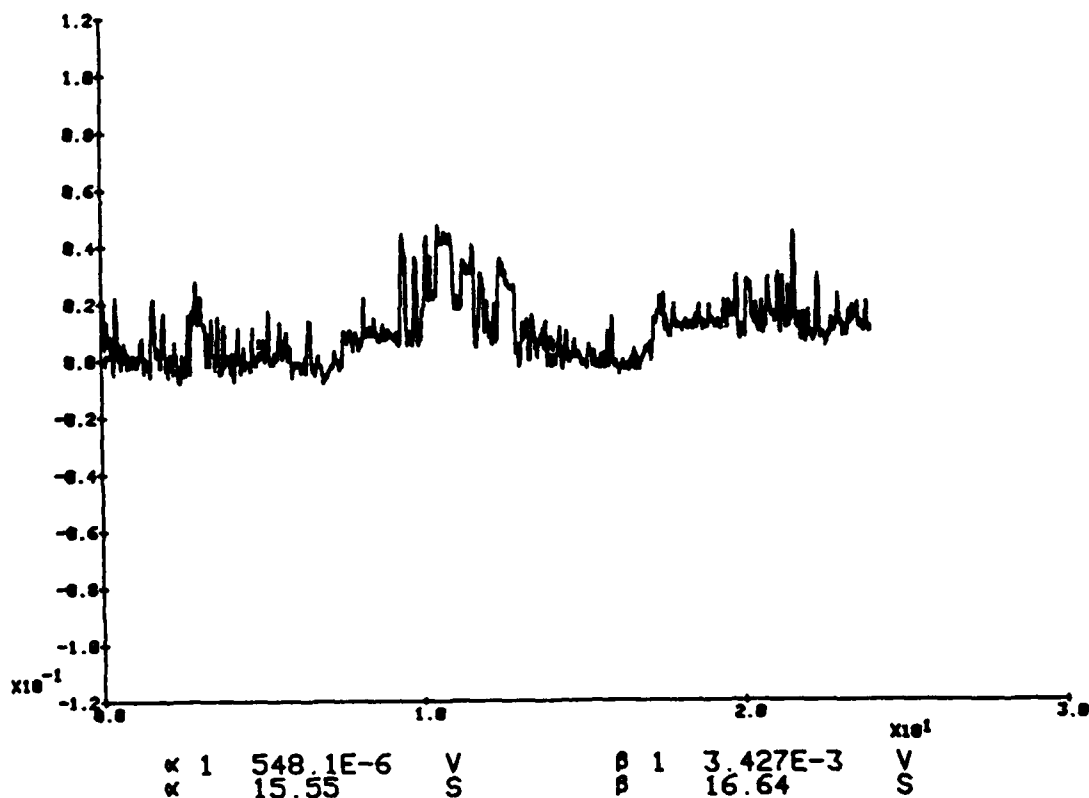
(Figure 17). This experiment was performed with 6-mM HEPES buffered medium containing 114-mM NaCl and 100 mM KCl. Although the fluctuations indicate cationic flux, it is uncertain whether the ion is sodium, potassium, or both. Amphotericin B produces general instability of the membrane along with discrete, channel-like events. This can be seen with the constantly drifting baseline and eventual rupture of the membrane, especially with voltage steps in excess of 150 mV.



Panel A represents current fluctuations across a phospholipid (phosphatidylcholine) bilayer membrane in the presence of d-tubocurarine and gramacidin. The holding voltage was 150 mV with a 50-mV command step to induce this dramatic, sustained activity. Noise was 0.9 RMS and the low-pass filter was set at 500 Hz. Rapid step-like fluctuations appear to be triggered by the presence of curare which combines with the existing gramacidin channel events.

Panel B is a trace of the same membrane structure before the addition of d-tubocurarine. The same step voltage reveals discrete steps in the baseline, perhaps indicative of the participation of numerous gramacidin channels. Note the usual gramacidin channel-like events riding on the shifted baseline. These shifts were not seen with d-tubocurarine.

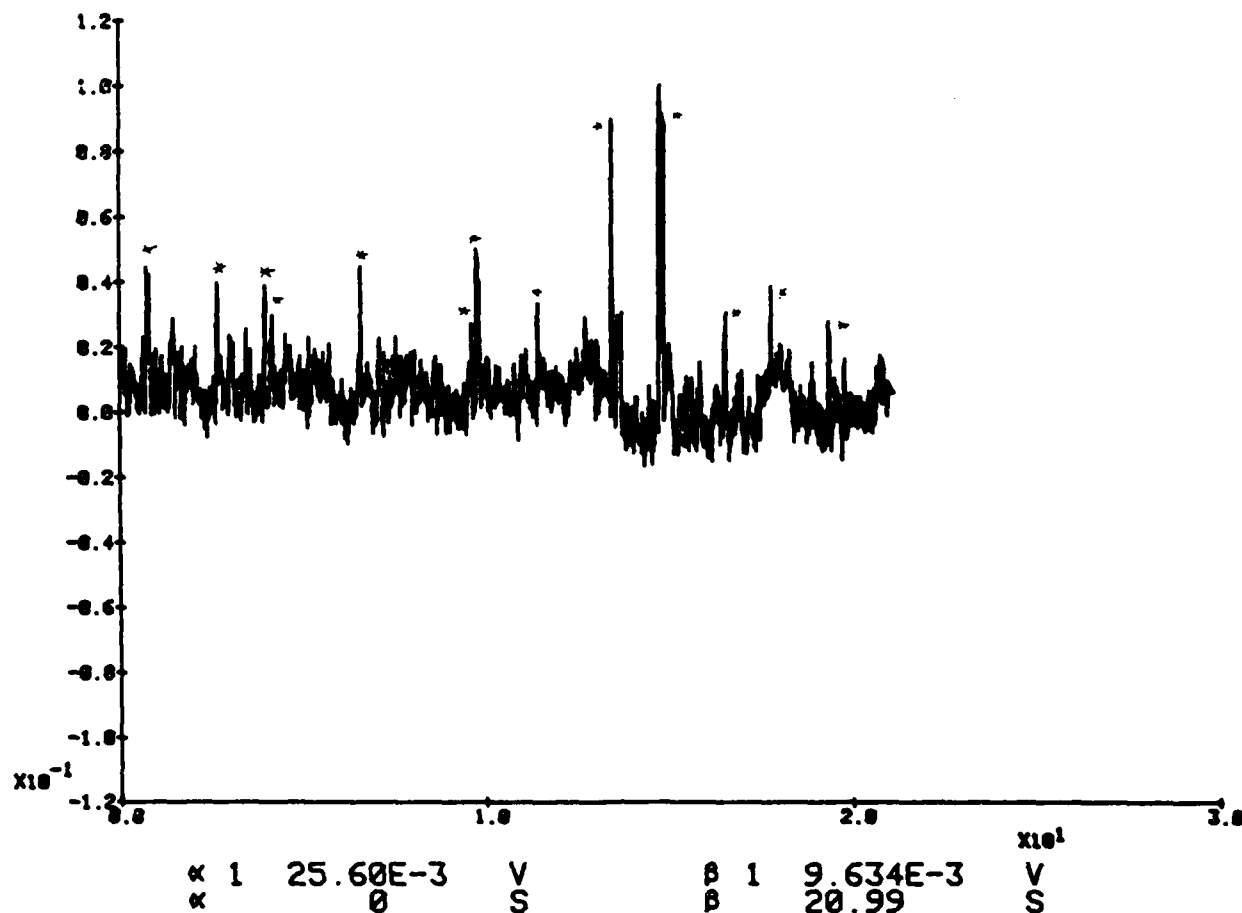
Figure 12. Transmembrane Conductance Changes in the Presence of d-tubocurarine and Gramacidin



Curare-induced conductance alterations across a patch phospholipid bilayer in the presence of gramacidin. This tracing of 24 s is taken from another experiment similar to Figure 12. Curare was added to the bath of the microprobe system in which gramacidin was previously introduced. Random, rapid fluctuations in transmembrane current were observed in addition to the normal gramacidin channels.

Figure 13. Curare-Induced Conductance Alterations Across a Patch Phospholipid Bilayer in the Presence of Gramacidin

The series of figures are representative of a number of successful experiments performed at room temperature (18-22 °C). Failure of the patch membrane is generally accompanied by major current fluctuations across the membrane similar to that shown in Figure 15, either spontaneously or in response to a command voltage step. For the best results, we found that membranes composed of soy lecithins or synthetic compounds such as diphytanoyl-phosphatidyl choline were most stable at room temperature. In addition, ultrafiltered solutions and conservative concentrations of "seeding" agents produced the clearest, most consistent, discrete channel-like events with a signal-to-noise ratio ideal for computer analysis.



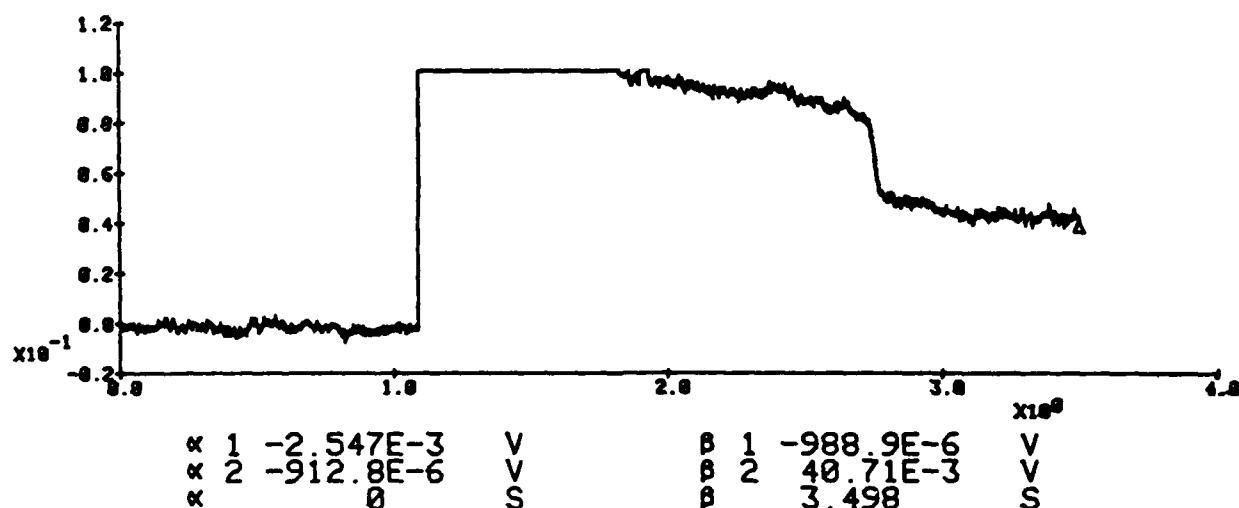
This is a 21-s tracing (3000 data points) of transmembrane conductances across a phospholipid bilayer in which only curare was present in the bath. The experimental design was similar to that in Figure 13. Asterisks mark what appeared to be discrete curare-induced channel-like events in the membrane. Noise was approximately 1.1 RMS; output gain was  $10 \times$ ; holding potential was 150 mV with a +50-mV command step, yielding 200 mV. These events were not transient, but continued throughout the recording period of about 30 min.

Figure 14. Curare-Induced Discrete Fluctuations in Membrane Conductance

#### 4.3 Cholinergic Drug Trials.

Curare was used originally as an arrow poison, then clinically for its neuromuscular blocking properties. Block comes primarily from dTC's antagonism with the nicotinic receptor at skeletal myoneural junction. It affects muscarinic receptors only at very high concentrations. Surprisingly, dTC induced spontaneous higher frequency, shorter duration step fluctuations in the patch membrane. These fluctuations appeared to be separate channel-like events which were not coupled to the gramicidin events (Figures 12, 13, and 14). It is uncertain if curare is a channel-former, whether unknown contamination induced the activity, or if the gramicidin molecules altered their behavior in the presence of the drug. The presence of dTC events in the

absence of gramacidin (Figure 14) strengthens the arguments that these events are intrinsic to the curare extract. These preliminary observations will serve as a precaution to planned studies with the muscarinic receptor with regard to the observation that agonists or antagonists may exert independent transmembrane conductance changes.



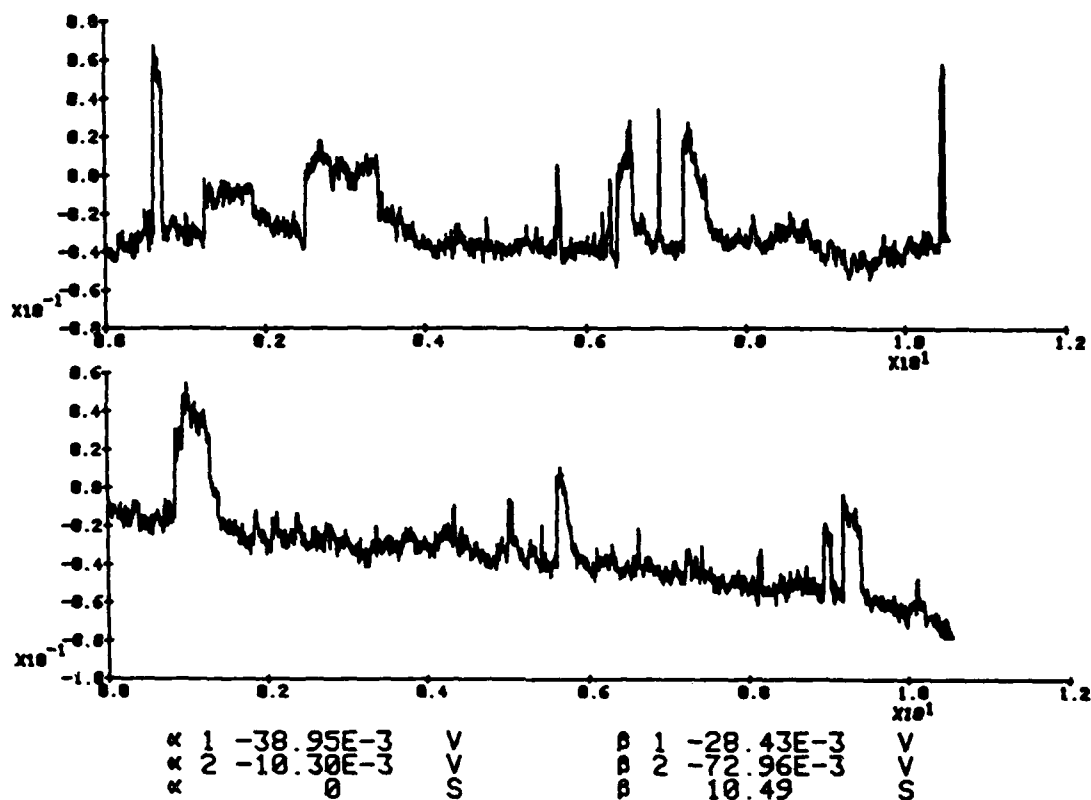
The artificial membrane was formed from Sigma lecithin: sphingomyelin standard (5:1) dissolved in hexane in order to spread the monolayer. Nonactin was applied at a concentration of 1 mg/mL to the bath side of the recording chamber. Holding potential was +150 mV; tracing was on autozero with output gain of  $5 \times$ ; headstage gain of 1. The cis and transmedia contained 200 mM KCl. This tracing illustrates the sudden, massive conductance shifts experienced with nonactin. Note the absence of channel-like activity in this 3.5-s sweep and a resetting of the baseline current after the large conductance change.

Figure 15. Conductance Shifts Induced by Nonactin Across a Patch Phospholipid Bilayer

#### 4.4 Sources and Study of Anion Channels.

In two of the experiments described in section 3.7, liposomes, formed by sonicating the protein-rich extracts from rat hemidiaphragm muscle with soy lecithin in n-hexane, were exposed to a patch membrane preformed with phosphatidyl choline: sphingomyelin:cholesterol (10:2:1). In one trial, discrete transmembrane conductances were observed that were opposite in direction from the gramacidin  $K^+$  channels observed earlier and of shorter duration (Figure 18). The fluctuations look very similar to the chloride channel events illustrated in Sakmann and Neher<sup>14</sup>. Because the medium contained only KCl and because of the reversed direction of the event, it was strongly suspected that a chloride channel had been detected. Despite repeated attempts, this activity could not be replicated. Retrospection of this problem directed our attention to purer preparations to be found in cell cultures. Three immediate sources were identified: cultured cardiac myocytes in Dr. Buxton's laboratory (University of Nevada,

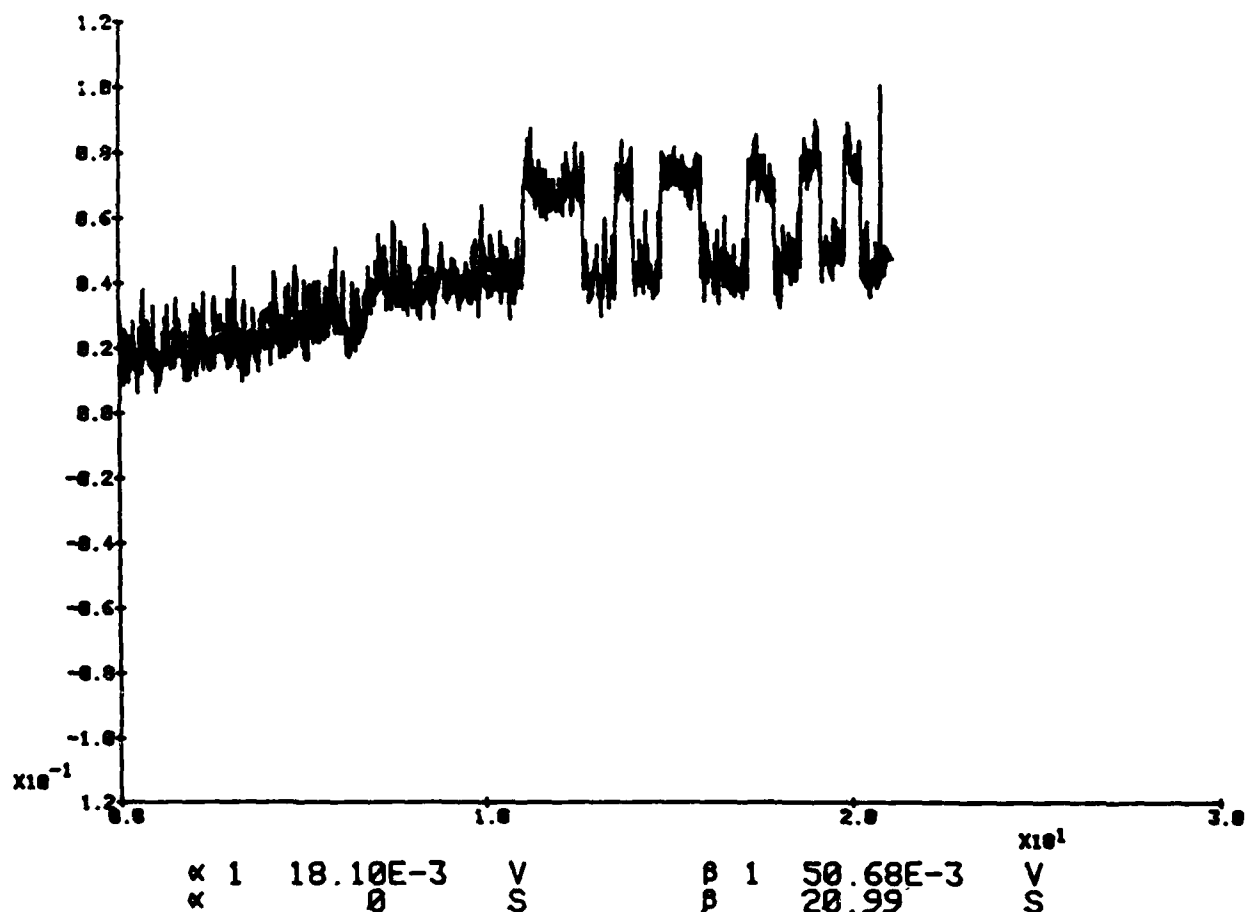
Reno, NV), cultured lymphocytes and "cancer" target cells in Dr. Redelman's laboratory (University of Nevada, Reno, NV), and cultured gastrointestinal smooth muscle cells in Dr. Sanders' laboratory (University of Nevada, Reno, NV).



The phospholipid bilayer was formed from monolayers of phosphatidic acid. In contrast to the previous figure, nonactin induced step-like conductance changes resembling the openings and closings of pores; however, with considerable irregularity in the duration as demonstrated by these tracings. The conductance changes were not discrete square-wave events exhibited by gramicidin or biological ion channels. These events generally have a delayed decay phase as the conductance returns to baseline. The baseline also drifts with less evidence of discrete steps or the massive changes observed in Figure 15. In a biosensor device, discrimination will be required to differentiate false signals which generate false signals which generate paranormal conductance or capacitance changes from true signals. The analysis programs associated with the microprobe system could differentiate between the events in this figure as compared to the gramicidin events in Figure 10 using the histogrammatic methods shown in Figures 4 and 5.

Figure 16. Nonactin-Induced Conductance Changes in Artificial Patch Membranes

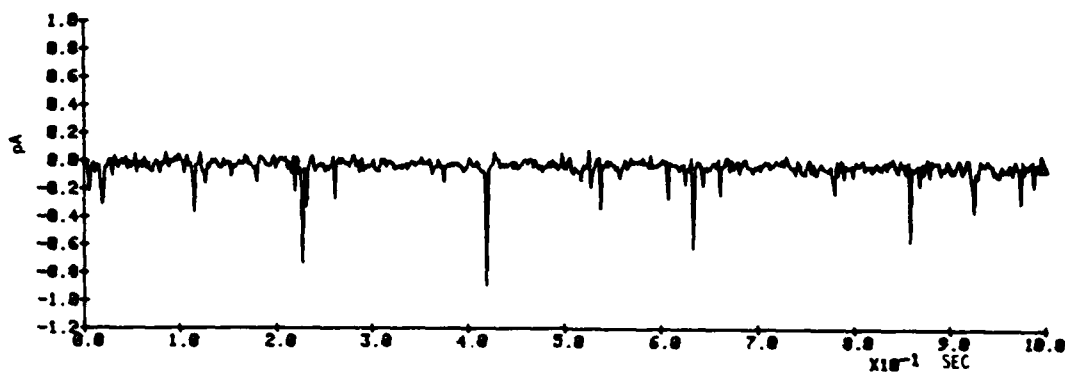
Research in Dr. Redelman's laboratory at the University of Nevada revealed a novel source of anionic channels likely to be identified as chloride channels. These occur in cultured cancer cell lines such as P815 (H-2d mastocytoma of DBA/2) and EL4 (H-2b thymoma of C57B23/6). When these target cells are attacked by cytotoxic T lymphocytes or by "cytolysin" prepared from the granules of rat tumor, large conductance changes occur in the cell membrane, leading to cellular disruption and cytocide.



The membrane was formed from monolayers of phosphatidylcholine and cholesterol (4:1) dissolved in pentane. A physiological buffered medium was used in this experiment: 6-mM HEPES; 114-mM NaCl and 100-mM KCl (20 °C). Amphotericin was introduced via the cis (intrapipette) route. The holding voltage was +200 mV. Spontaneous rectangular events are shown in this 21-s acquisition. These events are of greater magnitude (>1 pA) and longer duration (>500 ms) than the gramicidin channels. No discrete transmembrane events were observed with this compound below a holding voltage of 60 mV to zero. Note the characteristic drifting of the baseline in addition to the discrete fluctuations.

Figure 17. Voltage-Dependent Conductance Fluctuations Across Patch Phospholipid Membranes in the Presence of Amphotericin B

Theories explored by Dr. Redelman include the insertion of an ion conductive "pore" into the membranes of the target cells by the killer T cells. Further, the cytotoxic T lymphocytes may induce membrane component changes intrinsic to the target cell such as opening existing channels. Regardless of the precise mechanisms, the results are the same. An uncontrolled ion flux occurs in the target cells shortly after contact with the cytotoxic T cells. It was beneficial to attempt to identify the mechanism of cell-mediated target cell lysis since it likely involved a ready-made insertable "pore" or a preexisting ion (perhaps anionic) channel harvestable from the cultured cells. The goal was to isolate the entity responsible for the dramatic changes in membrane conductance for testing in our microprobe system. Outside circumstances forced the postponement of this work.



Membranes were constructed from lecithin: sphingomyelin: cholesterol (10:2:1) dissolved in pentane. Sonicates of a crude protein fraction of homogenized rat hemidiaphragm formed liposomes which were introduced via the trans-side of the bilayer. The channels observed in this 1-s trace appear to be anionic channels similar to chloride channels. These discrete fluctuations were less than 1 pA in magnitude and occurred spontaneously at a holding voltage of 100 mV. Autozero was used to reset the baseline to zero for this recording; the 500-Hz low-pass filtered was activated.

Figure 18. Unverified Chloride Channel Activity in Reconstructed Patch Membranes

#### 4.5 Activity of Acetylcholinesterase on Silicon Monoxide Substrates.

A pilot study was begun to determine if isolated acetylcholinesterase could be bound to the silicon monoxide substrate surface on silica wafers and still retain substantive enzymatic activity. One box of 64 silicon monoxide substrates was received at the end of April from the PL Program Manager, Mr. J.G. Wall, Jr. General information concerning the preparation (silanizing) of the surface for enzyme binding was received from A.L. Newman (Johns Hopkins University, Columbia, MD), Dr. J. Valdes (CRDEC), and Drs. R. Taylor and J. Chambers (University of Texas, San Antonio, TX). We have conducted seven trials to date. Our preliminary results are encouraging; three of seven experiments yielded moderate enzymatic activity.

#### 4.6 Muscarinic Receptors on Cardiac Myocytes.

An objective of this project in the coming year is to exploit the muscarinic acetylcholine receptor (mAChR) for use in the patch microprobe system. The source of this receptor will be cultured adult rodent cardiac myocytes from the laboratory of Dr. Iain Buxton, Assistant Professor of Pharmacology. Binding of ACh to the cardiac muscarinic receptor activates a potassium (K) channel that slows pacemaker activity. Delays in the time course of activation suggest a multistep process involving a second messenger. Coupling of the mAChR to the inward-rectifying K channel has been shown to require intracellular GTP.<sup>15</sup>

GTP-binding protein appears to regulate the function of the ionic channel without acting through cyclic nucleotide second messengers. The mAChR is also coupled to increased formation of inositol 1,4,5-triphosphate on stimulation with agonists such as carbachol. Dr. Buxton is conducting research into the coupling of the muscarinic and the  $\alpha$ -1 receptor to cyclic nucleotides and G-proteins. His goal is to study the K-channel activity of the mAChR in the isolated, cultured cardiac myocytes using patch-clamp technology. This work will increase our understanding of the mAChR mechanism already cited.<sup>15</sup>

It is noteworthy that the muscarinic receptor appears to be more complicated than the nicotinic receptor (nAChR). In the nAChR, a single protein molecule binds the neurotransmitter and also acts as an ion channel. However, in the mAChR, the K<sup>+</sup> channel (which is regulated by the mAChR) appears to be a distinct macromolecule likely coupled to the GTP-binding proteins. Thus, isolation and reconstitution of an active, coupled complex are likely to be more tedious than the nAChR. Agonist or antagonist binding verification studies in detergent-solubilized mAChR reveal the retention of the neurotransmitter binding sites, but do not confirm the successful reconstitution of the coupled K<sup>+</sup> channel.<sup>16</sup> The patch microprobe system will be ideal for testing the presence of K<sup>+</sup> channel activity in response to muscarinic receptor-agonist interaction as studies progress in this area.

## 5. CONCLUSIONS

The expertise in Black Lipid Membrane (BLM) technology has been transferred to a more refined microprobe system with the aim of providing a biosensor test system for research and development of a threat agent detection system. The patch microprobe system is an exquisitely sensitive electrophysiological methodology for measuring the transduction of electrical signals in biomembranes and transmembrane ionic conductances. The system has been computerized for data acquisition and analysis. Procedures have been established for manufacturing appropriate patch micropipettes utilizing an inexpensive glass capillary tubing (Fisher Blue-Tip hematological capillary glass) without micro-forging.

A technique for forming planar phospholipid bilayers across the tip of a micropipette has been established. The bilayer of predetermined phospholipid composition is assembled by multiple excursions through the interface of a phospholipid monolayer floating on aqueous medium. In keeping with the aim of simplicity for eventual routine testing, miniature recording chambers have been designed as flow cells. Inexpensive, disposable plastic sample cups have also been adapted for use in membrane manufacture and recording. The method as implemented has been useful in the testing of lipid compositions and performance of artificial membranes in environments of differing



temperatures, ionic strengths, solvents, voltages, and under the influence of selected chemical agents.

The electrical characteristics of test membranes have been augmented by the insertion of antibiotic compounds known to form ionic channels or to alter the conductance of the planar lipid bilayer. Gramacidin was discovered to be the most useful tool because it is an ionic channel-former and fails to function if the lipid membrane is not a bilayer, thus confirming true bilayer formation. Several appropriate phospholipid mixtures have been determined to be suitable for subsequent reconstitution work. Phospholipids of soy, plant, or synthetic origin proved to be more stable than lipids from animal extracts. Of particular usefulness to reconstitution efforts will be mixtures of soy lecithin and the synthetic phospholipid, L-alpha-diphytanoyl-lecithin. Addition of the antioxidant alpha-tocopherol to phospholipids of animal origin prompted stability in the patch membranes by inhibiting the autooxidation of the vital polyunsaturated fatty acid chains of the phospholipids.

Reconstitution trials with muscle homogenates and sonicates demonstrated the feasibility of extracting protein-rich fractions from mammalian tissue and reintroducing it via liposomes or vesicular sonicates (micelles) to an artificial membrane. These trials also reinforced the goal of working with simple, definable systems in developing strategies for reconstituting detector molecules in the membrane. Receptors or receptive materials must be isolated and purified because crude sonicates may contain large amounts of contaminants that will also exhibit channel properties. Another unexpected finding came from the work with cholinergic drugs. Commercially available curare was found to induce consistent channel-like events in patch membranes, thus increasing caution for future work with receptor agonists and antagonists. Studies considering the source of chloride channels directed attention away from muscle sonicates into purer systems such as cultured cancer cell lines, smooth muscle, or cardiac muscle. Plans were established to work with cultured cardiac myocytes in an attempt to isolate the muscarinic acetylcholine receptor for study in the patch microprobe system.

Examination of the receptor-channel activity will first be accomplished with conventional whole-cell patch-clamp methods using muscarinic agonists and antagonists. The next phase will be an attempt to transfer the active receptor complex to an artificial membrane. This is of considerable interest, because it will determine the capacity to isolate and to reconstitute not only a neurotransmitter binding site but also the associated K<sup>+</sup> channel complex.

The results of these experiments will be compared with the detection capabilities of the muscarinic receptor bound to the silicon monoxide substrates if this proves to be feasible. Thus, it is recommended that the patch microprobe system be further developed and employed in concert with the capacitance film

technology for the testing and selection of receptors for the biosensor R&D program. This will also provide a proven parallel technology, if biophysical limitations in the capacitance film technology prompt a multimethodological merger in the final biosensor detection device.

The accomplishments of this first year of study include the development and implementation of a computerized patch microprobe system for the electrophysiological study of membrane structures and potential detection receptors exhibiting regulatory influences on transmembrane conductances. This system will be useful in testing biological receptors for their ability to detect and discriminate selected threat agents in a biosensor scheme. This system will also be crucial in the comparative evaluation of biosensor detection schemes utilizing the capacitance-altering properties of biological receptor materials from membranes bonded to silicon monoxide wafers.

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